Selective Down-Regulation of Glioma-Associated Oncogene 2 Inhibits the Proliferation of Hepatocellular Carcinoma Cells

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Abstract
The sonic hedgehog (Shh) pathway contributes to the initiation and progression of tumors with various origins when aberrantly activated. In this study, we investigated if the Shh pathway is important for the proliferation of hepatocellular carcinoma (HCC) cells and also began to identify which components of the pathway play a pivotal role in the biology of HCC. Expression levels of components in the pathway were measured, and glioma-associated oncogene (Gli) 2 levels were found to be considerably higher in human HCC lines compared with normal liver. Gli2 levels were also higher in tumor tissue from HCC patients compared with normal liver. Antisense oligonucleotides (ASO) were used to specifically down-regulate Gli2, and this led to decreased proliferation of various HCC cell lines. However, inhibition of Gli1 and Gli3 with ASOs did not decrease proliferation in most HCC cell lines and inhibitors targeting the upstream components of the pathway, including smoothened (Smo), displayed antiproliferative effects in only a subset of HCC cell lines. Moreover, in cancer cells harboring Smo mutations or unresponsive to the Smo inhibitor 3-keto-α-aminoethylaminoethylcaproyldihydrocinnamoyl cyclopamine, the Gli2 ASO was still able to inhibit proliferation. The importance of Gli2 in HCC proliferation was further confirmed by the changes in expression of various HCC cell lines. Relative expression levels of each Gli gene in mediating downstream target genes through a family of zinc finger transcription factors, the glioma-associated oncogenes (Gli). However, other mechanisms, including overexpression of sonic hedgehog (Shh) and Gli3, loss of function of suppressors, such as Ptc and Sufu, and gain of function of the activator Smo, are also responsible for the activation of this pathway in cancer (7, 11, 12).

Introduction
As an important determinant in normal embryonic development, the hedgehog pathway plays crucial roles in tissue patterning, cell differentiation, and cell proliferation (1). Although the pathway remains inactive in most normal mature tissues, except during tissue repair, it can contribute to the development of various pathophysiologic conditions, including cancer, when aberrantly activated (2, 3). Recently, a growing list of evidence has indicated constitutive activation of the signaling pathway in cancers with gastrointestinal origins, such as in prostate (4–6), pancreatic (7), gastric (8), and colon (9, 10). Typically, the signaling pathway is initiated by the binding of ligands (sonic, indian, and desert hedgehog) to the receptor patched (Ptc), which in turn relieves the repression of the receptor on smoothened (Smo), a seven-transmembrane signaling protein. Smo then triggers a series of intracellular events, resulting in the regulation of numerous downstream target genes through a family of zinc finger transcription factors, the glioma-associated oncogenes (Gli). However, other mechanisms, including overexpression of sonic hedgehog (Shh) and Gli3, displays unique and sometimes overlapping roles in modulating the Shh pathway in a manner that is cell type or developmental stage dependent (13–16). The formation of hepatocellular carcinoma (HCC) is a multistep disease process driven by chronic inflammation and tissue injury from various pathogens and chemicals (17). Although several risk factors for HCC development are known, therapeutic options for the disease are very limited. It has been reported recently that, as in other gastrointestinal cancers, the Shh-Gli pathway might be important in HCC (18–20). However, studies addressing the role of specific Shh pathway component(s) for the proliferation and survival of HCC cells have not yet been reported.

In this study, we investigated the role of specific Shh pathway components in HCC cell lines. Relative expression levels of each component in the pathway in multiple HCC cell lines were determined. Additionally, the role of each Gli gene in mediating HCC cell proliferation and phenotype was addressed by specifically down-regulating their expression with antisense oligonucleotides (ASO). Finally, the phenotypic effects caused by Gli down-regulation were compared with the effects of 3-keto-α-aminoethylaminoethylcaproyldihydrocinnamoyl cyclopamine (KAAD-cyclopamine), a specific inhibitor of Smo. Our results indicate that HCC cells depend on the Shh-Gli pathway for their survival and that suppression of Gli2 activity inhibits HCC proliferation in multiple HCC cell lines, including lines that are resistant to inhibitors targeting the upstream components of the pathway.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Materials and Methods

Cell culture, animals, and reagents. Various human HCC or colon cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA) and cultured following the provider’s instructions or as otherwise indicated. Transgenic mice with SV40 t/T-driven HCC were described previously (21) and purchased from Taconic (Germantown, NY). KAAD-cyclopamine, a Smo inhibitor, and its inactive analogue tomatidine were purchased from Taconic (Germantown, NY). Desferrioxamine B, a chelator for iron, was purchased from Sigma (St. Louis, MO), and 3,4-dihydroxyphenylalanine (Dopamine) was purchased from Toronto Research Laboratory (Toronto, Ontario, Canada) and Sigma (St. Louis, MO), respectively. Shh-blocking antibody and recombinant N-Shh were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and R&D Systems (Minneapolis, MN), respectively.

ASOs, small interfering RNA, plasmids, and transfection. 2′-Methoxyethyl–modified chimeric ASOs were used throughout this study and synthesized as described previously (22). Active ASOs targeting each Gli gene were identified by screening 50 to 100 ASOs designed against each gene to determine which ASOs were most effective at reducing target mRNA as determined by Taqman real-time reverse transcription-PCR (RT-PCR) analysis (22). The efficacy of these ASOs was confirmed in concentration–response experiments, and only the most effective ASOs for each gene were used in further experiments. HCC cells were plated in 96-well plates (5–10,000 per well), 6-cm dishes (400–600,000 per dish), or 10-cm dishes (1 × 10^5 to 2 × 10^6 per dish) 16 h before transfection. ASOs were transfected into cells by Lipofectamine (3 μg/mL per 100 mmol/L oligonucleotide; Invitrogen, Carlsbad, CA) in Opti-MEM medium (Invitrogen) at the indicated concentration for 4 h, and then, the medium was switched into complete growth medium. Small interfering RNA (siRNA) for Gli2 and cyclinophilin B control (the sequences are in Supplementary Table S1) were purchased from Dharmacon (Lafayette, CO) and transfected into SNU423 cells using DharmaFECT transfection agent. The expression vectors for human Gli1 and wild-type Smo were purchased from OriGene Technologies, Inc. (Rockville, MD). The human Gli2 (j) vector was kindly provided by Dr. D. Markovitz (University of Michigan, Ann Arbor, MI), and the pCMV-Gli3 plasmid was obtained from Dr. Phil Iannaccone (Northwestern University, Chicago, IL). The vectors were transfected into the cells along with pEGFP using LipofectAMINE 2000 (Invitrogen) at the ratio of 1:2 (DNA/lipid) for 5 h or Fugene 6 (Roche Applied Sciences, Indianapolis, IN) at 1:3 overnight, and the medium was switched to complete growth medium.

Immunohistochemical analysis of Gli2. Human HCC tissue arrays were purchased from US Biomax, Inc. (Rockville, MD). Liver was taken either from 3-month-old SV40 transgenic mice developing HCC or from C57BL/6 wild-type animals (The Jackson Laboratory, Bar Harbor, ME). Formalin-fixed paraffin sections of mouse liver or the slides from HCC tissue arrays were subjected to deparaffinization and rehydration in xylene and graded alcohol. Hydrogen peroxide treatment (0.3%) was done for 30 min to quench endogenous peroxidase activity, and then, the slides were blocked with 5% normal donkey serum (Chemicon International Inc., Temecula, CA) for 30 min and subsequently incubated with rabbit polyclonal Gli2 antibody (5 μg/mL; Abcam Ltd., Cambridge, United Kingdom) overnight at 4°C. Biotin-labeled donkey anti-rabbit IgG (The Jackson Laboratory) at 1:200 and 3,3′-diaminobenzidine were applied for 45 and 5 min each. Finally, the slides were counterstained with hematoxylin, dehydrated, and mounted for microscopy.

Reporter gene assay. Six tandem repeats containing a consensus Gli-binding site were synthesized and cloned into the pG3L-promoter reporter vector (Promega, Madison, WI). Cells (100–150,000 per well) were plated on collagen-coated 12-well plates, and the plasmid (1 μg/well) along with the Renilla vector (0.5 μg/well) were delivered into the cells by LipofectAMINE 2000. Luciferase activity was measured by using the Dual-Luciferase Reporter Assay System (Promega) and represented as fold induction over pG3L-promoter. Variation in transfection efficiency was corrected by normalizing the firefly luciferase activity to the Renilla luciferase activity.

RT-PCR and quantitative real-time RT-PCR. Total RNA was isolated by using Qiagen RNeasy 96 BioRobot 9604 kit for the 96-well plate cultures or RNeasy Mini kit for the large-scale cultures following DNase I treatment. RNA from normal human adult or fetal liver was purchased from Cell Applications, Inc. (San Diego, CA). RT-PCR was done with One-Step RT-PCR kit (Qiagen), and the PCR products were separated on an agarose gel. The sequences of the primers are provided in Supplementary Table S1. Quantitative real-time RT-PCR was done by ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA). The abundance of mRNA of each gene was normalized to the amount of total RNA determined by Ribogreen (Invitrogen).

Cell proliferation and apoptosis assays. Cells (5–10,000 per well) were seeded on collagen-coated 96-well plates 16 h before treatment. Cell proliferation from the transfection with ASOs or treatment with the inhibitors was measured after 72 h of incubation by using Promega CellTiter 96 AQuneous One Solution. Triplicates or quadruplicates of wells were used for each treatment, and the experiments were repeated at least twice. The plates were read at 450 nm on a SpectraMax spectrophotometer (Molecular Devices Corp., Sunnyvale, CA). Apoptosis of cells was assessed typically 36 h after treatment by using Apo-ONE Caspase-3/7 Assay kit (Promega) according to the supplier's directions. To measure cell proliferation by the bromodeoxyuridine (BrdUrd) incorporation assay, HCC cells were plated on the slide chambers coated with poly-L-lysine (20–40,000 per well). Following various treatments for 48 h, the cells were chased with 10 μmol/L BrdUrd for 60 min (Hep3B) or 90 min (SNU423) before fixation and processed by using Roche BrdUrd labeling and detection kit 1. BrdUrd-positive cells were detected under green light, and at least 400 cells were counted for each treatment. Apoptotic cells among the green fluorescent protein–positive ones were also visualized by 4′,6-diamidino-2-phenylindole staining under UV microscope as described previously (23).

Electrophoretic mobility shift assay. SNU423 cells were transfected with 100 nmol/L of Gli1 or Gli2 ASOs, and nuclear extracts were prepared as described previously (24). The oligonucleotides (5′-CTTCTCCGGGTGGTGC-CCGGCTTG-3′) were end labeled with [γ-32P]ATP by using T4 polynucleotide kinase (Amersham Biosciences, Piscataway, NJ), purified with Sephadex G-25 spin column (Amersham Biosciences), incubated with 4 μg of nuclear extract at room temperature for 25 min, and separated on 5% nondenaturing PAGE (∼0.25 Tris-borate EDTA), dried, and exposed to X-ray film at −80°C overnight.

Immunoblotting. The cells transfected with ASOs or treated with KAAD-cyclopamine were lysed in radioimmunoprecipitation assay buffer containing a protease inhibitor cocktail (Roche Applied Sciences), 0.5 mM/L sodium orthovanadate, 10 mM/L β-glycerophosphate, and 1 mM/L NaF. Whole-cell lysates (40 μg) were separated by PAGE, transferred to a nitrocellulose membrane, and immunoblotted with the primary antibodies against E-cad (BD Biosciences, San Diego, CA) at 1:500, cyclin D1 (BD Biosciences) at 1:500, cyclin B1 (BD Biosciences) at 1:500, E2F1 (Santa Cruz Biotechnology) at 1:200, Foxm1 (Abcam) at 1:100, Bcl-2 (BD Biosciences) at 1:500, Ptk1 (Santa Cruz Biotechnology) at 1:200, p21 (Santa Cruz Biotechnology) at 1:200, Gli1 (Rockland, Gilbertsville, PA) at 1:400, Snail (Santa Cruz Biotechnology) at 1:200, poly(ADP-ribose) polymerase (PARP; BD Biosciences) at 1:500, caspase-3 (Alexis, San Diego, CA) at 1:250, and α-tubulin (Sigma) at 1:3,000 in 0.05% Tween 20–TBS (T-TBS) containing 5% skim milk overnight at 4°C followed by washing with T-TBS for 30 min. Goat anti-mouse or rabbit IgG coupled with horseradish peroxidase (Bio-Rad Laboratories) were used as secondary antibodies at 1:3,000 in T-TBS with 5% skim milk at room temperature for 1 h followed by washing with T-TBS for 30 min. Immunospecific bands were detected with the Enhanced Chemiluminescence Plus detection kit (Amersham Biosciences).

Detection of Smo mutation. Genomic DNA was either isolated from HCC cells using Genomic DNA Isolation kit (BioVision, Mountain View, CA) or purchased from Biochain Institute, Inc. (Hayward, CA) for normal human heart and liver tissues. PCR amplification was done using PfuUltra High-Fidelity DNA polymerase (Stratagene, La Jolla, CA) for 12 human Smo exons, and the amplicons were directly sequenced (Retrogen, Inc., San Diego, CA).

Results

Differential expression of Shh-Gli pathway genes in HCC. To determine the relative expression of Shh-Gli pathway components in HCC cell lines compared with normal liver, RT-PCR was done
with RNA from normal fetal and adult liver tissues, a SV40 T-antigen–transformed human hepatocyte cell line (THLE-2), and nine human HCC cell lines (Fig. 1A). Although all of the components of the pathway were expressed in HCC lines to different extents, Ptc1, Gli1, and Gli2 levels were much higher in many HCC lines relative to normal liver. This differential expression was especially true for Gli2, which was almost undetectable in both fetal and adult liver, although its expression level was markedly higher in THLE-2, 7721, SK-Hep1, SNU398, SNU423, and SNU449. High level of Gli2 expression in HCC tissues was further shown by immunohistochemical analysis of human HCC samples and the livers from a mouse model of HCC (21) driven by SV40 Tag expression. In samples from human patients, Gli2 immunoreactivity was much stronger in tumor sections compared with patient-matched normal liver sections (Fig. 1B, left). In the mouse model of HCC, Gli2 immunoreactivity was also much stronger in the tumor nodules compared with normal mouse liver (Fig. 1B, right). We also found that the HCC cell lines with high Gli2 expression (7721, SK-Hep1, SNU398, SNU423, and SNU449) showed the characteristics of poorly differentiated HCC, expressing low levels of differentiation-related genes (Supplementary Fig. S1). In contrast, HepG2, Hep3B, and Huh7 cells with low Gli2 expression seem to belong to a well-differentiated group. These results suggest that Gli2 might be involved in the dedifferentiation and invasion of HCC cells.

**Down-regulation of Gli2 leads to the inhibition of cell proliferation in HCC.** Because Gli2 was highly expressed in HCC, we next explored the roles of Gli2 in HCC cell proliferation by specifically down-regulating its expression by applying an antisense approach in SNU423 and Hep3B cells. We chose these cells as representative examples of poorly and well-differentiated HCC cell lines, respectively. When determined by real-time quantitative RT-PCR, Gli2 mRNA levels were reduced by up to ~90% following ASO treatment in a concentration-dependent manner. Control oligonucleotide treatment had little effect on Gli2 gene expression (Fig. 2A). The specificity of the Gli2 effect was further confirmed by looking at the expression of Gli1 and Ptch1, two known downstream target genes within the Shh-Gli pathway (Fig. 2B). Gli2 mRNA levels were already very low 8 h after transfection, and the

![Figure 1](image_url)

**Figure 1.** Expression of Shh pathway genes in HCC lines and tissues. A, one-step RT-PCR was done to detect the expression of Shh, Smo, Ptc1, Gli1, Gli2, and Gli3. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) level is for normalization purposes. Note that Gli2 is almost undetectable both in fetal and adult human livers. B, Gli2 expression detected by immunohistochemistry is shown in patient-matched representative samples of HCC and nontumor tissues (left) or from the livers of a murine HCC model or strain-matched wild-type mice (right). Much stronger Gli2 staining was observed in tumor compared with normal liver.
Gli2 target genes (Ptch1 and Gli1) were subsequently down-regulated and maintained at low levels for 48 h. Due to the fact that there is no reliable commercially available Gli2 antibody for immunoblotting, we could not assess Gli2 down-regulation at the protein level. However, considering the short half-life of Gli proteins (~40 min) and the labile nature of Gli2 (25, 26), it is not surprising to see the rapid down-regulation of Gli1 and Ptch1 mRNA following the knockdown of Gli2 mRNA expression. Finally, Gli2 down-regulation led to an increase in caspase-3/7 activity and a decrease in cell proliferation both in SNU423 and Hep3B cells, whereas the control oligo and total RNA was isolated at the indicated times. RT-PCR was done to detect the expression levels of Gli2, Gli1, and Ptch1. GAPDH level is shown for normalization purposes. C, caspase-3/7 activity was measured 30 to 36 h after transfection and is shown as fold induction over untransfected cells. D, cell proliferation was assessed 72 h after transfection and represented as percentage of untransfected cells. Columns, mean of triplicate or quadruplicate samples; bars, SD. Each study was repeated at least thrice.

Figure 2. Specific down-regulation of Gli2 leads to the decrease in expression of downstream target genes, the inhibition of cell proliferation, and an increase in apoptosis in HCC cells. SNU423 and Hep3B cells were plated on three sets of collagen-coated 96-well plates (5,000–10,000 per well) and transfected with increasing concentrations of Gli2-specific ASOs. Control oligo was also included to show the specificity of the Gli2 ASO. A, total RNA was isolated 36 h after transfection and Gli2 levels were determined by real-time RT-PCR. Data as percentage of untransfected cells (UTC). B, SNU423 cells were transfected with the Gli2 ASO along with the control oligo and total RNA was isolated at the indicated times. RT-PCR was done to detect the expression levels of Gli2, Gli1, and Ptch1. GAPDH level is shown for normalization purposes. C, caspase-3/7 activity was measured 30 to 36 h after transfection and is shown as fold induction over untransfected cells. D, cell proliferation was assessed 72 h after transfection and represented as percentage of untransfected cells.
Gli2 is the dominant regulator among the Gli transcription factors in the Shh-Gli signaling pathway in HCC cells.

Effects of down-regulation of Gli genes and KAAD-cyclopamine treatment on cell proliferation in various HCC cell lines. To determine if the importance of Gli2 in cell growth and proliferation is restricted to a few cell lines or is more general in various HCC types, we tested the effects of Gli down-regulation on cell proliferation in additional HCC lines by using ASOs targeted to each Gli gene. Although the degree of inhibition varied from cell type to cell type, Gli2 down-regulation resulted in a significant decrease in cell proliferation in all HCC cell lines tested, whereas Gli1 or Gli3 ASOs had little effect (Fig. 4A). Next, we tested whether attempting to block the Shh-Gli signaling pathway with KAAD-cyclopamine, a selective Smo inhibitor that acts at the cell surface, could affect the proliferation of HCC cells. Whereas Hep3B, SK-Hep1, and SNU998 cell proliferation was significantly reduced by the inhibitor (>30% inhibition) compared with the inactive analogue tomatidine, SNU423, SNU449, and Huh7 were affected either marginally or not at all (Fig. 4B). Because Smo was expressed at similar levels in all cell lines (Fig. 1A), this suggests that some HCC cell lines are resistant to the inhibitor. The importance of down-regulating Gli2 expression in blocking the entire Shh-Gli signaling pathway in HCC cells was further confirmed by combined treatment of cells with Gli2 ASO and KAAD-cyclopamine. Adding the Smo inhibitor to SNU423 cells transfected with Gli2 ASO did not enhance antiproliferative effects of the ASO alone, even when cell proliferation was measured by the more sensitive BrdUrd incorporation assay (Fig. 4C). On the other hand, combined treatment in Hep3B cells resulted in a larger decrease in proliferation than with either single treatment alone. These results again suggest that Gli2 is the main mediator of the signaling pathway but that inhibition at other levels of the pathway can be effective in some HCC cell lines.

Differential effects of Gli2 modulation and KAAD-cyclopamine on the expression of downstream target genes in SNU423 and Hep3B cells. Although both SNU423 and Hep3B cells were inhibited in their proliferation by Gli2 ASOs, only Hep3B cells responded to KAAD-cyclopamine. Thus, to elucidate the molecular basis for this differential response to the two inhibitors, we looked at the expression levels of downstream target genes. We particularly focused on the genes directly involved in cell proliferation, apoptosis, and differentiation. Although many genes have been identified as Gli targets in several studies, most of them were from overexpression experiments (28, 29). Therefore, it was necessary to see if those genes are regulated by Gli under more normal circumstances and, if so, how differently they behave in response to the Gli ASOs. Cells were either transfected with the ASOs or treated with KAAD-cyclopamine for 48 h before immunoblots were done. A known Gli target gene, the antiapoptotic protein Bcl-2, was selectively down-regulated by the Gli2 ASO in SNU423

![Figure 3](https://www.aacrjournals.org/doi/fig/10.1158/0008-5472.CAN-06-3180.0008.png)

**Figure 3.** Shh pathway is functionally active in HCC, and Gli2 is a major regulator of the pathway. SNU423 and Hep3B cells were plated in collagen-coated 12-well plates (100,000 per well), transfected first with a 6xGli-binding site luciferase vector, (Gli)6, along with the pRL-TK for 24 h. Next, the cells were transfected with 100 nmol/L of control oligo or ASO for Gli1, Gli2, or Gli3 for 16 h, and then, luciferase activity was determined. A, down-regulation of each Gli gene by the ASO was confirmed by real-time RT-PCR in SNU423 cells. B, reporter activity was normalized to Renilla luciferase and is shown as fold induction over pGL3-transfected cells. C, gel shift assay was done with the nuclear extracts from Gli ASO-transfected SNU423 cells in the presence of radiolabeled oligonucleotides containing consensus Gli-binding site. EMSA, electrophoretic mobility shift assay.
Figure 4. Differential proliferation responses of HCC cells to the down-regulation of Gli1 and to the Smo inhibitor KAAD-cyclopamine. 

A, cells were plated on 96-well plates and transfected with multiple ASOs for each Gli gene at 50 and 100 nmol/L. Cell proliferation was measured 3 d after transfection and expressed as percentage relative to untransfected cells. 

B, cells were treated with increasing concentrations of KAAD-cyclopamine or inactive analogue tomatidine in complete growth medium [5–10% fetal bovine serum (FBS)]. Cell proliferation was determined on day 3 and represented as the percentage of untreated control cells. Columns, mean of triplicate samples; bars, SD. Data are representative of two to three independent experiments.
cells, whereas the ASOs for Gli1, Gli3, or KAAD-cyclopamine had no effect on Bcl-2 (Fig. 5A). This result is very consistent with the recent report that Bcl-2 is preferentially regulated by Gli2 over Gli1 in breast cancer and HaCaT cells (30, 31). As reported previously (32), Hep3B cells did not express a detectable level of Bcl-2 protein.

c-Myc has been known to be a key player in carcinogenesis especially in HCC development (33). The protein was down-regulated by both Gli2 ASO and KAAD-cyclopamine in Hep3B cells, whereas, in SNU423, only the Gli2 ASO was able to strongly reduce c-Myc protein levels. The two well-known Gli targets, Gli1 and Ptch1, were also down-regulated by Gli2 ASO in SNU423 cells, whereas KAAD-cyclopamine did not alter the Gli1 or Ptch1 protein levels significantly. Interestingly, Gli1 protein levels rebounded back to basal levels 48 h after transfection, suggesting that there might be a strong positive feedback loop as reported previously (4, 34).

Up-regulation of Snail is one of the important changes in gene expression when tumors undergo epithelial-mesenchymal transition, thus becoming more invasive and metastatic (35–37). Snail levels were higher in poorly differentiated SNU423 cells than in Hep3B cells as expected. The Snail protein level was down-regulated by Gli2 ASO transfection but not by KAAD-cyclopamine. In this regard, Gli2 was a key regulator in breast cancer metastasis (38). It has been reported recently that p21, a cyclin-dependent kinase inhibitor, is up-regulated by Gli1 siRNA and cyclopamine in gastric carcinoma cells (8). In fact, p21 levels increased slightly by Gli1 ASO in SNU423 cells, but the induction of protein was more robust by the Gli2 ASO and especially by KAAD-cyclopamine. Despite the induction of p21 by both Gli1/Gli2 ASOs and KAAD-cyclopamine in SNU423 cells, it is unlikely that the protein was solely responsible for mediating the antiproliferative effect because KAAD-cyclopamine failed to inhibit cell proliferation in SNU423 cells and p21 levels were too low to be detectable in Hep3B (39).

However, the level of another cell cycle inhibitor, p27, was increased only by the Gli2 ASO in SNU423 cells, whereas the protein was significantly induced both by the Gli2 ASO and KAAD-cyclopamine in Hep3B cells. In contrast to the results in other cancer types (40), cyclin D1 was not down-regulated by either Gli ASOs or KAAD-cyclopamine as reported in a recent HCC study (20). Instead, Gli1 and Gli3 ASOs increased cyclin B1 levels, whereas Gli2 ASO or KAAD-cyclopamine had no effect. Other genes identified as Gli downstream target genes through overexpression experiments, such as Foxm1b and E2F1, were not changed.

Therefore, it seems that many downstream target genes of the pathway are differentially regulated by each Gli transcription factor in different types of cancers. Taken together, these results show that down-regulation of Gli2 shifts HCC cells to a more proapoptotic (by down-regulating Bcl-2 and c-Myc), antiproliferative (by down-regulating c-Myc and Gli1 and up-regulating p21 and p27), and differentiated state (by down-regulating Snail). This new gene expression pattern results in the inhibition of cell proliferation and the promotion of apoptosis.

Because down-regulation of Gli genes or treatment with the Smo inhibitor displayed different effects on gene expression and cell proliferation in HCC cells, we next investigated their roles in Shh signaling by ectopically overexpressing each Gli gene in KAAD-cyclopamine–responsive Hep3B cells. First, the transactivation potential of each Gli was assessed by reporter gene assay. Whereas Gli1 and Gli2 induced to a similar extent the activity of a luciferase reporter containing consensus Gli-bindingsites in its promoter, overexpression of Gli3 resulted in a decrease in luciferase activity (Fig. 5B). However, when individual endogenous genes were investigated, only Gli2 significantly increased the levels of c-Myc and Bcl-2 (Fig. 5C), suggesting the importance of promoter context in Gli-mediated gene expression. Furthermore, overexpression of
Gli2 was able to inhibit the cell death induced by KAAD-cyclopamine as shown by the decrease in PARP and caspase-3 cleavage, surrogate markers of apoptosis. In accordance with the results from down-regulation experiments of the Gli isoforms, these data suggest that each Gli has a preference for different downstream targets and the genes involved in the proliferation and survival (c-Myc and Bcl-2) might be primarily regulated by Gli2 at least in HCC cells.

**Down-regulation of Gli2 overcomes the resistance to the inhibitor KAAD-cyclopamine in cells carrying mutations in Smo.** The finding that some HCC cell lines are resistant to the Smo inhibitor was reminiscent of reports of cell lines with activating mutations in Smo, which lead to high activity of the Shh-Gli pathway. This prompted us to investigate if Smo mutations confer the resistance to KAAD-cyclopamine seen in some of the HCC cell lines by sequencing the exons of Smo in SNU423 and Hep3B (data not shown). However, no mutations were found, suggesting that Smo may not be directly responsible for the resistance of SNU423 cells to KAAD-cyclopamine. Next, we investigated if down-regulation of Gli2 can be effective even in cells known to contain Smo mutations. Because there is no report available on the status of Smo mutations in HCC cell lines, we took advantage of the earlier report in colon cancer cells, where Smo mutations were detected by single-strand conformational polymorphism analysis (41). DLD1 and HCT15 cells contain a mutation in exon 11 of the Smo gene, which is not found in the normal population. When the expression levels of the hedgehog components were measured by RT-PCR, Gli2 was found to be expressed in both colon cancer cells at a similar level (Fig. 6A). Ptch1, a signature gene of activated hedgehog pathway, was also detected. When the activity of the hedgehog pathway was measured using the reporter gene assay, a modest increase in luciferase activity was observed (Fig. 6B), suggesting that the Shh pathway remains active in these colon cancer cells. Finally, the effects of KAAD-cyclopamine or Gli down-regulation by the ASOs on cell proliferation were compared (Fig. 6C). Whereas the Smo inhibitor had no effect on cell proliferation in either cell line, Gli2 down-regulation led to a decrease in cell proliferation, more significantly in DLD1 cells. Furthermore, the addition of KAAD-cyclopamine to the cells transfected with Gli2 ASO did not increase the antiproliferative effects from Gli2 down-regulation (Fig. 6D). These results show that Gli2 down-regulation could be an effective treatment in some cases of cancer that would be unresponsive to Smo inhibition by KAAD-cyclopamine.

**Discussion**

Activation of the Shh-Gli pathway has been reported in various cancer types, including HCC. However, each cancer type responds...
differently to currently available inhibitors of the pathway depending on their mutation status and the expression profile of the components (5, 42). Moreover, identification of a growing list of proteins which can modulate the Shh-Gli signaling pathway by directly interacting with the major components or through the modification of pathway proteins, has shown that the pathway is more complex than originally thought (43–45). Despite the potential activation of the pathway at multiple steps, the gene expression changes are ultimately manifested by Gli transcription factors, the final regulators of the pathway. Therefore, specifically down-regulating the expression of these transcription factors might be a preferred approach for blocking the signaling pathway.

Here, we have shown that HCC cell lines express all the components of the Shh pathway, albeit to different extents. We also identified Gli2 as a key regulator in the proliferation and survival of HCC cells. Despite their essential functions in mediating Shh-Gli signaling, the relative roles of each Gli protein in carcinogenesis have never been evaluated directly especially in human gastrointestinal cancers. Our findings in this study are novel in that, unlike in other types of cancer, Gli2 played the dominant role over Gli1 or Gli3 in regulating the expression of target genes and the proliferation of HCC cells. Moreover, the fact that down-regulation of Gli1 alone was not sufficient to inhibit the proliferation of HCC cells and had little effect on the expression of downstream target genes, whereas down-regulation of Gli2 decreased both Gli1 and other target gene levels, suggests that Gli2 may directly regulate the expression of many downstream genes. However, there is still the possibility that Gli1 may be involved in the proliferation of some cases of HCC as observed in other types of cancers (18). Overexpression of Gli2 was reported in prostate and breast cancer cells; however, its implication in carcinogenesis has not been as well characterized as Gli1 or Gli3 (6, 25, 46). It has been reported recently that Gli2 is degraded by the ubiquitin-proteasome pathway (25), suggesting that stabilization of Gli2 protein could contribute to the overexpression of this protein in certain types of cancer. Among the Gli members, Gli1 possesses only an activator domain, whereas Gli2 contains both activator and repressor domains, and Gli3 mostly functions as a repressors (47). Therefore, it is very possible that Gli proteins would not be expected to share a completely common set of genes as their downstream targets. Some of the genes identified as Gli1 targets in one cancer type were not changed in their expression in other cancer types, suggesting

Figure 6. Down-regulation of Gli2 inhibits the proliferation of cancer cells containing Smo mutations. A, RNA from HCT15 and DLD1 was subjected to RT-PCR for Shh, Ptc1, Smo, Gli1, Gli2, and Gli3 genes. GAPDH level is shown for normalization purposes. B, a luciferase vector with 6xGli-binding site (Gli)6 was transfected into colon cancer cells for 24 h and the Gli reporter activity is represented as fold induction over pGL3. C, colon cancer cells were either transfected with 100 nmol/L ASOs for each Gli gene or treated with 10 μmol/L of KAAD-cyclopamine or tomatidine. D, Gli2 ASO or control oligonucleotides were transfected into the cells in the presence of KAAD-cyclopamine or tomatidine. Cell proliferation was determined at 72 h and is shown as percentage of untransfected cells.
that Gli3 can have a preference for different targets in different cancer types (28, 31). In fact, a recent array study following the overexpression of Gli1 and Gli2 lacking the repressor domain (Gli2ΔN) revealed significant differences in the expression of their targets, both quantitatively and qualitatively (31). Although more complete array studies are warranted to identify other genes that are differentially altered in their expression after Gli2 down-regulation, significant changes in the levels of several important genes in carcinogenesis, including down-regulation of c-Myc and Bcl-2 and up-regulation of p27 as reported in our study, would be sufficient to explain why HCC cells were inhibited in their proliferation following Gli2 down-regulation. Moreover, in our study, c-Myc and Bcl-2 levels increased significantly only when Gli2 was overexpressed and not when Gli1 was overexpressed. In this regard, it will be worthwhile to reassess the roles of Gli2 in other types of cancers where only Gli1 was evaluated extensively. This can be accomplished by knocking down the expression of each Gli gene individually with ASOs or siRNAs. Data generated from these experiments will help to elucidate the relative roles of each Gli in the development and progression of cancers with different origins.

The fact that several known risk factors for HCC development, including alcohol, hepatitis virus B/C, and aflatoxin B, lead to a damaging inflammatory response supports the notion that some cancers are the outcome of repeated chronic tissue injury/repair processes (48) and that HCC might be a result of this process. Considering the strong activation of the Shh-Gli pathway in various tissues insulted with pathogens and chemicals, it will be important to know when and which cell type(s) in liver show activation of this pathway in the course of HCC progression. In this regard, increases in the levels of some of Shh-Gli components in cirrhotic livers are important in that HCC often contains cirrhotic lesion (49, 50). The relevance of blocking Shh-Gli pathway to treat HCC can be further supported by the evidence that the pathway can cross-talk with the Wnt/β-catenin and Notch signaling pathways, two well-known contributors of HCC development (51–53).

We have also shown that Gli2 down-regulation could overcome the unresponsiveness of some HCC lines to Shh antibody or the Smo inhibitor KAAD-cyclopamine. These results suggest that there may be ligand-independent activation mechanisms and/or frequent epigenetic changes at Smo or its downstream components in HCC as seen in other types of cancers, thus limiting the usefulness of these potential therapeutic compounds in treating various types of HCC. Importantly, the fact that some cells displayed resistance to KAAD-cyclopamine despite the lack of detectable mutations in Smo strongly suggests possible additional mutations at components between Smo and Gli. Although we did not identify mutations in any of the components leading to the constitutive activation of the pathway or conferring resistance to the Smo inhibitor in two HCC lines we used extensively in this study (SNU423 and Hep3B), a recent study showing a Smo mutation in a HCC patient suggests possible mutations at components of the pathway in HCC (20). Therefore, the chance that HCC patients could display resistance to inhibitors that target upstream components of the pathway is likely. The target of our ASO, Gli2, acts at the downstream terminus of the pathway. Therefore, we wanted to see if it would be useful in blocking the proliferation of cancer cell lines that are known to harbor mutations in an upstream component of the pathway. Because the reports of mutations in this pathway in HCC are in patients and not HCC cell lines, we used colon cancer cell lines that were reported to contain mutations in Smo. In these cell lines, suppression of Gli2 was indeed able to inhibit cell proliferation, whereas KAAD-cyclopamine had no significant effect. Taken together, our findings suggest that selective down-regulation of Gli2 expression by use of an ASO can block the hedgehog signaling pathway at the final step irrespective of upstream events and may be an attractive treatment option in HCC.

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