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Activation of the hedgehog pathway in human hepatocellular carcinomas

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Liver cancers, the majority of which are hepatocellular carcinomas (HCCs), rank as the fourth in cancer mortality worldwide and are the most rapidly increasing type of cancer in the United States. However, the molecular mechanisms underlying HCC development are not well understood. Activation of the hedgehog pathway is shown to be involved in several types of gastrointestinal cancers. Here, we provide evidence to indicate that hedgehog signaling activation occurs frequently in HCC. We detect expression of *Shh*, *PTCH1* and *Gli1* in 115 cases of HCC and in 44 liver tissues adjacent to the tumor. Expression of *Shh* is detectable in about 60% of HCCs examined. Consistent with this, hedgehog target genes *PTCH1* and *Gli1* are expressed in over 50% of the tumors, suggesting that the hedgehog pathway is frequently activated in HCCs. Of five cell lines screened, we found Hep3B, Huh7 and PLC/PRF/5 cells with detectable hedgehog target genes. Specific inhibition of hedgehog signaling in these three cell lines by smoothened (SMO) antagonist, KAAD-cyclopamine, or with Shh neutralizing antibodies decreases expression of hedgehog target genes, inhibits cell growth and results in apoptosis. In contrast, no effects are observed after these treatments in HCC36 and HepG2 cells, which do not have detectable hedgehog signaling. Thus, our data indicate that hedgehog signaling activation is an important event for development of human HCCs.

Abbreviations: DMEM, Dulbecco-modified essential medium; FBS, fetal bovine serum; HCCs, hepatocellular carcinomas; MTT, 1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan; PCR, polymerase chain reaction; RT–PCR, reverse transcription–polymerase chain reaction; SMO, smoothened; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

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Introduction

Liver cancer, with hepatocellular carcinoma (HCC) as the major tumor type, is a malignancy of worldwide significance (1–4). HCC ranks as the eighth cause of cancer-related death in American men with 14 000 deaths yearly and is the most rapidly increasing type of cancer in the United States (2). The medical oncology community is largely unprepared for this looming epidemic of HCC. Although the increase of HCC in the United States is correlated with the increasing prevalence of chronic infection with hepatitis C virus (HCV), the molecular understanding of HCC development remains elusive (2). A majority (70–85%) of patients present with advanced or unresectable disease, making the prognosis of HCC dismal, and systemic chemotherapy is quite ineffective in HCC treatment. The first essential step for development of effective therapeutic approaches is to identify specific signaling pathways involved in HCC.

The role of the hedgehog pathway in human cancers has been established through studies of basal cell nevus syndrome (BCNS) (5,6), a rare hereditary disorder with a high risk of basal cell carcinomas, and activation of the hedgehog pathway has been observed in other cancers such as prostate cancer and gastrointestinal cancers (7–17). Targeted inhibition of the hedgehog pathway results in growth inhibition in cancer cell lines with activated hedgehog signaling (10–17). The hedgehog pathway is essential for embryonic development, tissue polarity and cell differentiation (18). The hedgehog pathway is critical in the early development of the liver and contributes to differentiation between hepatic and pancreatic tissue formation, but the adult liver normally does not have detectable levels of hedgehog signaling (10,19). In this report, we characterize expression of sonic hedgehog and its target genes in 115 HCC specimens. The role of hedgehog signaling on cell growth is further demonstrated in five HCC cancer cell lines.

Materials and methods

Tissue samples

A total of 115 specimens of HCC tissues were used. Of these, 14 specimens were received as discarded materials from General Surgery of the Shan Dong Qi Lu Hospital, Jinan, China. Pathology reports and H&E stained sections of each specimen were reviewed to determine the nature of the disease and the tumor histology. The remaining 101 HCC specimens were from Sun Yat-Sen University. Forty-four liver tissues adjacent to the tumor were also included in this study. None of the patients had received chemotherapy or radiation therapy prior to specimen collection.

In situ hybridization

In situ hybridization was performed according to the manufacture's instructions (Roche Molecular Biochemicals, Indianapolis, IN) and our published protocol

(16,17). In brief, tissues were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) and embedded with paraffin. Then 6 μm thick tissue sections were mounted onto Poly-L-Lysine slides. Samples were treated with proteinase K (20 μg/ml) at 37°C for 15 min, refixed in 4% paraformaldehyde and hybridized overnight with a digoxigenin-labeled RNA probe (at a final concentration of 1 μg/ml). The hybridized RNA was detected by alkaline phosphatase-conjugated anti-digoxigenin antibodies (Roche Molecular Biochemicals, Indianapolis, IN), which catalyzed a color reaction with the substrate NBT/BCIP (Roche Molecular Biochemicals). Blue signal indicated positive hybridization. We regarded tissues without blue signals as negative. As negative controls, sense probes were used in the hybridization and no signals were observed. *In situ* hybridizations were repeated at least twice for each tissue sample with similar results.

RNA isolation and quantitative RT–PCR

Total RNA of cells was extracted using a RNA extraction kit from Promega according to the manufacturer (Promega, Madison, WI), and quantitative PCR analyses were performed according to a previously published procedure (17,20). Triplicate C_T values were analyzed in Microsoft Excel using the comparative $C_T(\Delta\Delta C_T)$ method as described by the manufacturer (Applied Biosystems, Foster City, CA). The amount of target ($2^{-\Delta\Delta CT}$) was obtained by normalization to an endogenous reference (18S RNA) and relative to a calibrator. We used the following primers for RT–PCR of *Shh*: forward primer—5′-ACCGAGGGCTGGGACGAAGA-3′; reverse primer—5′-ATTTGGCCGCCACCGAGTT -3′

Cell culture, transfection and drug treatment

HCC cell lines [Hep3B, HepG2, HCC36, PLC/PRF/5 (as PLC throughout this manuscript) and Huh7] were generously provided by Drs Chiaho Shih, Tien Ko and Kui Li at UTMB. All cells were cultured in Dulbecco-modified essential medium (DMEM) with 10% FBS and antibiotics. Cells were treated with 2 μM KAAD-cyclopamine, a specific antagonist of smoothened (SMO) (21) (dissolved in DMSO as 5 mM stock solution, Cat# K171000 from Toronto Research Chemicals, Canada), in 0.5% FBS in DMEM for indicated time mentioned in the figure legends. Previously, we performed toxicity assay with KAAD-cyclopamine in GI cancer cells and found that 10 μM of KAAD-cyclopamine can lead to non-specific toxicity (16). In fact, 5 or 10 μM KAAD-cyclopamine was quite toxic to cells regardless of hedgehog signaling status (our unpublished observation), and was, thus, not used in this study. Tomatidine (2 μM in 0.5% FBS DMEM, Sigma Cat# T2909), a structurally similar compound with non-specific inhibition on hedgehog signaling, was used as a negative control. In addition, the specific inhibition of hedgehog signaling in HCC cells was achieved by addition of Shh neutralizing antibodies (1 μg/ml in 0.5% FBS DMEM, Cat# SC-9024, Santa Cruz Biotechnology, Santa Cruz, CA). Most cell lines were treated with KAAD-cyclopamine (2μM) or Shh antibodies (1 μg/ml) in 0.5% FBS in DMEM medium for an indicated time (see figure legends for details). However, for Hep3B cells, we used 2% FBS in DMEM because Hep3B cells cannot grow in 0.5% FBS DMEM medium. Transient transfection of Gli1 in HCC cells was performed using LipofectAmine according to manufacturer's recommendation (Plasmid:LipofectAmine = 1:2.5). Cells with ectopic expression of Gli1 were subjected to drug treatment and to TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) assay.

Cell viability and TUNEL assays

For cell viability analysis, we used two methods: Trypan blue analysis and MTT assay. Trypan blue analysis was performed according to a procedure from the manufacturer (Invitrogen, CA) (22). The percentage of trypan blue positive cells (dead cells) was calculated under a microscope and triplicates of samples for each treatment were used. The experiment was repeated three times. MTT assay was performed using a previously published procedure (22). In brief, triplicates of samples for each treatment were used in a 96-well format. Twenty microliters of MTT (10 mg/ml in PBS) was added to each well (containing 100 μl cultured medium, 0.5% FBS DMEM in this study). Three hours later, medium was aspirated, and 100 μl of a mixture of isopropanol and DMSO (9:1) added into each well. Thirty minutes later, the 570 nm absorbance was measured with a microplate reader from Molecular Devices Co Sunnyvale, CA. BrdU labeling was for 1 h and immunofluorescent staining of BrdU was performed as reported previously (23). TUNEL assay was performed using a kit from Roche Biochemicals according to a published procedure (24). In brief, cells were fixed with 4% paraformaldehyde at room temperature for 1 h and permeated with 0.1% Triton X-100, 0.1% sodium citrate (freshly prepared) on ice for 2 min. After washing with PBS, each sample was incubated with 50 μl of TUNEL reaction mixture at 37°C for 30 min. TUNEL label solution (without enzyme) was used as a negative control. TUNEL positive cells were counted under a fluorescent microscope. The counting was repeated three times, and the percentage from each counting was calculated.

Statistical analysis

Statistical analysis was performed by Binomial proportions analysis. The association of mRNA transcript expression with various clinicopathological parameters was also analyzed; a P-value < 0.05 was considered to be statistically significant.

Results

Expression of PTCH1 and Gli1 in primary HCC

In order to assess hedgehog signaling activation in HCC, we assayed *PTCH1* and *Gli1* expression in 115 cases of HCC specimens. As the target genes of the hedgehog pathway, expression of *PTCH1* and *Gli1* transcripts indicate hedgehog signaling activation (25,26). Primarily, we used *in situ* hybridization to assess hedgehog signaling activation in our collected tissues ($n = 115$), which was further confirmed in selected specimens by real-time PCR. The results are summarized in Table I.

For *in situ* hybridization analysis, blue signal was regarded as detectable expression of the target. Tissues without blue signals were regarded as negative for the target. Using *in situ* hybridization, 79 of 110 (70%) tumor specimens had detectable expression of *Gli1* (representative images are shown in Figure 1A, and summarized in Table I, with additional images and data provided in Supplementary Table 1 and Supplementary Figures 1–6), indicating that *Gli1* expression is detectable in many HCCs. The sense probe gave no detectable signals (Figure 1A), confirming the specificity of *in situ* hybridization in our experiments. In most cases, *Gli1* expression was detectable in the tumor nest, not in the adjacent liver tissue (Figure 1A; Supplementary Figure 1 and Table 1) or in the stroma (arrows in Figure 1A).

In comparison with the *Gli1* transcript, the *in situ* hybridization signal of *PTCH1* was generally less intense (Figure 1B and Supplementary Figures 1–6), but 56% (60 of 107) of HCC specimens were positive for *PTCH1* transcript. We found a total of 51 tumors (out of 98 informative HCCs) (52%) with detectable expression of both *Gli1* and *PTCH1* (Table I, Supplementary Table 1), which suggests activated hedgehog signaling in these specimens. Our analysis indicates that activation of hedgehog signaling (as indicated by expression of both *Gli1* and *PTCH1* transcripts) occurs more frequently in HCC than in the adjacent liver tissue (Table I, Supplementary Table 1 and Supplementary Figure 1). There are several cases in which only *Gli1* or *PTCH1* was expressed (Supplementary Table 1), suggesting that expression of *Gli1* and *PTCH1* may be differentially regulated. Further analysis of our data did not reveal association of the hedgehog signaling activation with tumor size or tumor differentiation (Table I). Tumors with hepatocirrhosis were not significantly different from tumors without hepatocirrhosis in the expression of *Gli1* and *PTCH1* (Table I).

In situ hybridization data was further confirmed by real-time PCR in several tumor specimens in which 70% of the tissue mass was actually tumor tissue (Figure 1C and D). Consistent with *in situ* hybridization, expression of *Gli1* and *PTCH1* were detectable in the tumor, not in the adjacent liver tissue in most cases (will be discussed later in the Discussion). Our data indicate that expression of *Gli1* and *PTCH1* in the tumor was 3- to 30-fold higher than that in adjacent liver tissues (Figure 1C and D). The real-time PCR analyses further confirmed that activation of the hedgehog pathway is a common event in HCC.

Table I. Detection of *Shh*, *PTCH1* and *Gli1* expression in HCC and in adjacent liver tissue by *in situ* hybridization

	Shh			Hedgehog pathway activation						
				PTCH1		*Gli1*		Pathway activation		
	pos	neg	*P*-value	pos	neg	pos	neg	pos	neg	*P*-value
HCC	64/108	44/108	<0.01*	60/107	47/107	79/110	31/110	51/98	47/98	<0.01*
Adjacent tissues	5/41	36/41		18/43	25/43	15/44	29/44	9/43	34/43	
Tumor size										
Small (<3 cm)	16/31	15/31	0.316	17/31	14/31	25/32	7/32	16/31	15/31	0.896
Large (>3 cm)	46/74	28/74		42/74	32/74	52/75	23/75	35/66	31/66	
Tumor differentiation										
Well	34/52	18/52	0.107	30/51	21/51	43/52	9/52	29/51	22/51	0.264
Mod-poor	20/41	21/41		22/41	19/41	32/43	11/43	19/42	23/42	
Sex										
Male	47/81	34/81	0.651	43/81	38/81	58/83	25/83	35/72	37/72	0.258
Female	17/27	10/27		17/26	9/26	21/27	6/27	16/26	10/26	
Hepatocirrhosis										
+	14/19	5/19	0.163	14/20	6/20	14/20	6/20	11/17	6/17	0.251
–	49/87	38/87		43/83	40/83	63/87	24/87	39/79	40/79	

Statistical analysis was performed by Binomial proportions analysis. A P-value < 0.05 was considered to be statistically significant. The association of mRNA transcript expression with various clinicopathological parameters was also analyzed. Statistically significant difference was indicated by asterisk (*).

pos, positive signal; neg, negative signal; well, well-differentiated tumors; mod-poor, moderately to poorly differentiated tumors. Elevated expression of at least two hedgehog target genes was regarded as being positive (pos) in activation of the hedgehog pathway, whereas elevated expression of one hedgehog target gene was regarded as being negative (neg) in hedgehog signaling activation.

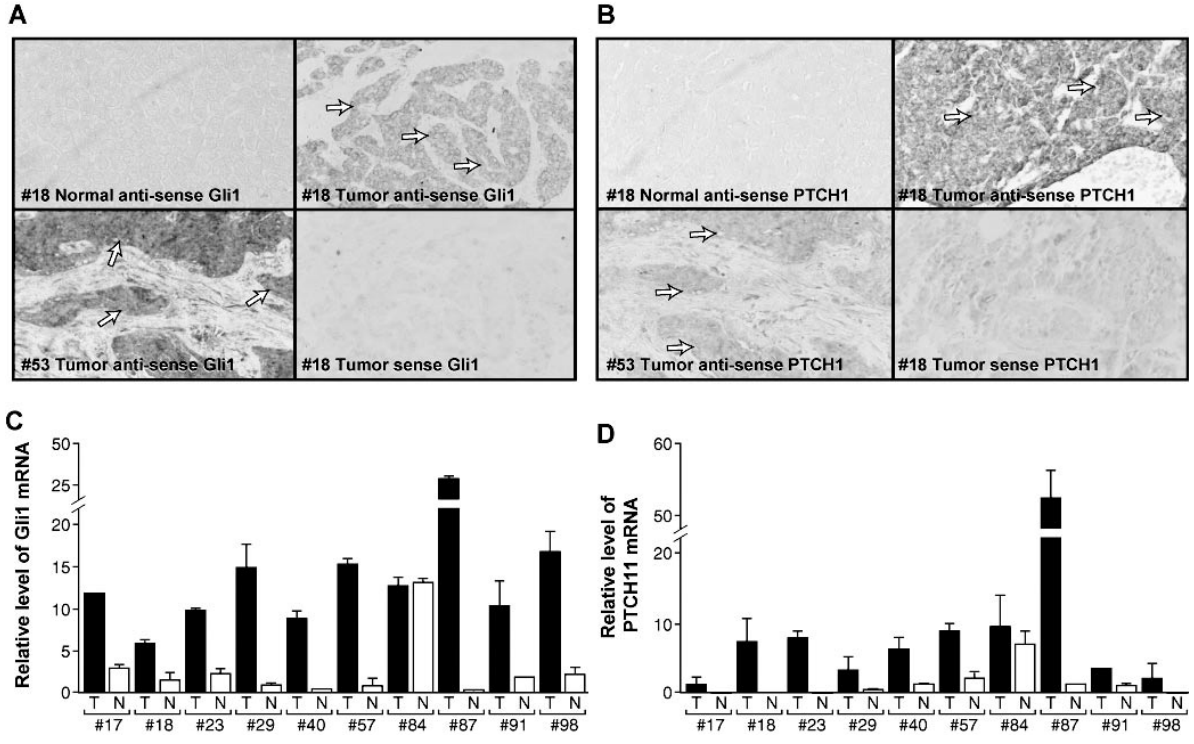

Fig. 1. Detection of *Gli1*, *PTCH1* expression in primary HCCs. *In situ* hybridization detection of *Gli1* (**A**) and *PTCH1* (**B**) transcripts in HCCs was performed as reported previously. Positive signals (dark grey staining) were observed in the tumor ('Tumor', tumor nests indicated by arrows), not in the stroma surrounding the tumor nests or in the liver tissue adjacent to the tumor ('Normal'). The sense probes did not give any positive signals (A and B), confirming the specificity of our *in situ* hybridization. Additional pictures have been included in the Supplementary Figures. Expression of *Gli1* and *PTCH1* was further confirmed by real-time PCR analysis done in triplicate (**C** and **D**) in selected tumor specimens in which 70% of the tissue mass was tumor tissue. Expression of *Gli1* (C) and *PTCH1* (D) from the tumor (T) was 3- to 30-fold higher than that from the adjacent liver tissue (N). Data indicates values relative to 18S RNA and to a calibrator. The data from this analysis are consistent with those from *in situ* hybridization analysis.

Expression of Shh in HCCs

To investigate if *Shh* is associated with hedgehog signaling activation in HCCs, *Shh* expression was first detected by *in situ* hybridization. We detected *Shh* transcripts in 64 of 108 HCC specimens, but not in the majority of liver tissues adjacent to the tumor (Figure 2A, Table I and Supplementary Figures 1, 4–6). *Shh* transcript was only detectable in the tumor nests, not in the stroma (dark grey signals in Figure 2A), suggesting that

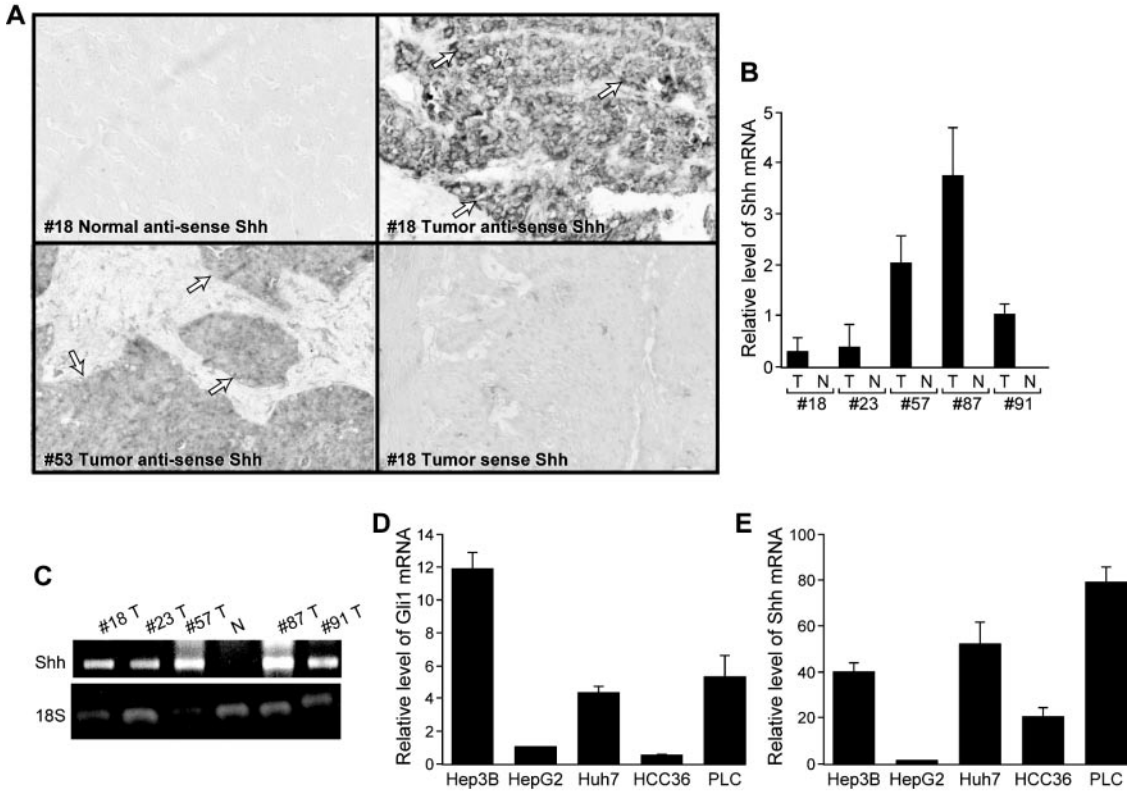

Fig. 2. Detection of *Shh* expression in HCCs. *In situ* hybridization (**A**), real-time PCR (**B**) and regular RT–PCR (**C**) were used to detect *Shh* transcript. *Shh* transcript (dark grey signals in A) resided in the tumor ('Tumor', tumor nests indicated by black arrows), not the stromal or adjacent liver tissue ('Normal') (A), suggesting that the tumor tissue is the major source for *Shh* expression. To confirm our *in situ* hybridization results, we used real-time PCR to detect *Shh* expression (B), which was further confirmed by RT–PCR (C). Shh transcripts were detected only in the tumor (T), not in the adjacent liver tissue (N). Tumors with detectable *Gli1* and *PTCH1* transcripts all had detectable *Shh*, suggesting a major role of *Shh* for activation of the hedgehog pathway in HCCs. Additional real-time PCR experiments showed a relatively high level of *Gli1* (**D**), *PTCH1* (not shown here) and *Shh* (**E**) in three HCC cell lines: Hep3B, Huh7 and PLC. Data indicates values relative to 18S RNA and to a calibrator.

cancer cells are the major source of *Shh* expression. Almost all tumors with detectable *Gli1* and *PTCH1* expression had detectable *Shh* transcript (Figures 1 and 2, Supplementary Table 1, Supplementary Figures 5 and 6). *Shh* expression in the tumor was further confirmed by real-time PCR and regular RT–PCR (Figure 2B and C). Thus, it appears that *Shh* induction may be the trigger for activated hedgehog signaling in HCCs. In support of this hypothesis, we detected expression of *Shh* in all three HCC cell lines with detectable transcript of *Gli1* (Figure 2D and E).

Targeted inhibition of hedgehog signaling in HCC cells

SMO is the major signal transducer of the hedgehog pathway; thus cancer cells with activated hedgehog signaling through *Shh* expression should be sensitive to treatment with the SMO antagonist, KAAD-cyclopamine (Toronto Research Chemicals, Cat# K171000, Toronto, Canada) (21). First, we screened HCC cell lines for hedgehog signaling activation by real-time PCR detection of *Gli1* and *PTCH1* and found that hedgehog signaling pathway was activated in Hep3B, PLC and Huh7 cells but not in HepG2 and HCC36 cells (Figure 2D shows the level of *Gli1* transcript). Addition of KAAD-cyclopamine (2 μM) greatly decreased the level of *Gli1* transcript in three cell lines (Hep3B, PLC and Huh7) (Figure 3A), whereas no changes on *Shh* expression were observed (Supplementary Figure 7). The closely related compound tomatidine, which does not affect SMO signaling and thus served as a negative control, had little discernible effect on hedgehog target genes. This data indicates specific inhibition of the hedgehog pathway by KAAD-cyclopamine in these cells.

As a result of inhibited hedgehog signaling by KAAD-cyclopamine treatment, we observed an inhibition on cell growth of Huh7 cells, but not on that of HepG2 cells (Figure 3B and C). The specificity of hedgehog signaling inhibition was further demonstrated using Shh neutralizing antibodies (Figure 3B and C). We found that addition of Shh antibodies at a concentration of 1 μg/ml reduced cell growth of Huh7 cells but had no effect on HepG2 cells (Figure 3B and C). Further analysis indicates that BrdU incorporation was also reduced after treatment with KAAD-cyclopamine in Huh7 cells (see Supplementary Figure 8).

Following treatment with KAAD-cyclopamine or Shh antibodies, we found that PLC cells underwent apoptosis whereas no apoptosis was observed in HepG2 cells (Figure 4A shows data from KAAD-cyclopamine treatment). Data from TUNEL assay was confirmed by Trypan blue staining (data not shown here). The percentage of apoptotic cells varied from cell line to cell line, with PLC being the most sensitive cell line (over 20% TUNEL positive cells after KAAD-cyclopamine treatment for 8 h, Figure 4B). Similar data were also observed after Shh antibody treatment (data not shown here). These data demonstrate that the HCC cells with activated hedgehog signaling are sensitive to targeted inhibition of the hedgehog pathway, whereas other HCC

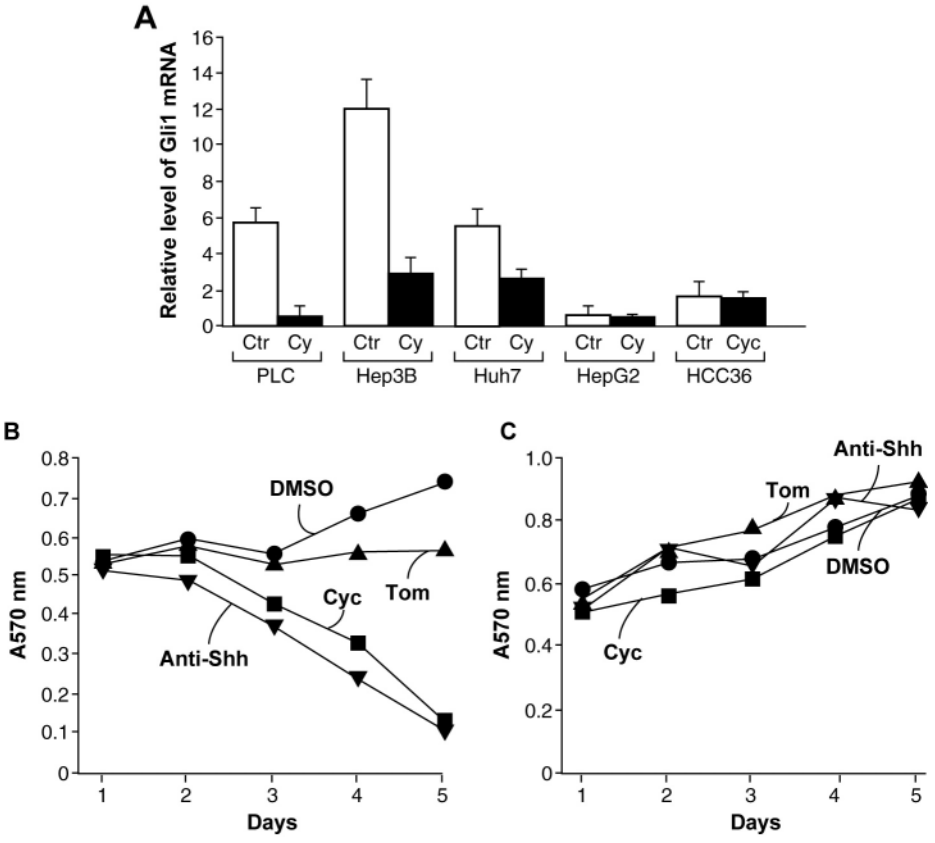

Fig. 3. Hedgehog signaling and growth of HCC cells. Real-time PCR data of *Gli1* transcript shows that in the presence of 2 μM KAAD-cyclopamine (**A**) or 1 μg/ml Shh neutralizing antibodies (data not shown here) for 12 h (see Materials and methods for details on drug-treatment conditions), the level of hedgehog target gene *Gli1* was decreased in the three cell lines with activated hedgehog signaling (PLC, Hep3B and Huh7). In contrast, no effects were observed in HCC36 and HepG2 cells, in which hedgehog signaling is not activated. Cell growth of Huh7 (**B**) and HepG2 (**C**) cell lines were examined by MTT assay. Huh7 cells were inhibited by 2 μM KAAD-cyclopamine (Cat# K317000, Toronto Research Chemicals) or 1 μg/ml Shh neutralizing antibodies (Cat# 9024, Santa Cruz Biotechnology) (Figure 2B). This inhibition was specific because addition of tomatidine, a structurally similar but non-specific compound for hedgehog signaling, did not affect cell growth. In contrast, cell growth of HepG2 was not affected by KAAD-cyclopamine (2 μM) or Shh neutralizing antibodies (1 μg/ml) (C), confirming the specific growth inhibition of HCC cells through targeted inactivation of hedgehog signaling.

cells (without activated hedgehog signaling) are resistant to these treatments.

Because KAAD-cyclopamine and Shh antibodies only affect signaling upstream of SMO, we hypothesize that cells with ectopic expression of the downstream effector Gli1 may prevent KAAD-cyclopamine-mediated apoptosis if these treatments are specific to the hedgehog pathway. In Huh7 cells, we transiently expressed Gli1 under the control of the CMV promoter (pLNCX vector) (23). After KAAD-cyclopamine treatment, we found that all Gli1-expressing cells ($n = 500$) were negative for TUNEL, demonstrating the specificity of KAAD-cyclopamine. Similarly, Gli1-expressing Huh7 cells were resistant to Shh antibody treatment (data not shown). This study also suggests that downregulation of *Gli1* may be an important mechanism by which targeted inhibition of hedgehog signaling mediates apoptosis in HCC cells.

Taken together, our findings indicate that activation of the hedgehog pathway is quite common in liver cancers. Expression of *Shh* and its target genes, *Gli1* and *PTCH1*, is more frequent in the tumor than in the adjacent liver tissue. This activation of hedgehog signaling is not associated with other clinicopathological parameters of the tumor. HCC cells with activation of the hedgehog pathway are sensitive to targeted inhibition of hedgehog signaling. These data support our hypothesis that activation of the hedgehog pathway is an important event in the development of HCC.

Discussion

Hedgehog signaling in liver cancer

Over 500 000 new cases of liver cancers are reported each year worldwide; most of them are HCCs. Most of HCC patients (70–80%) are diagnosed late in the progression of the disease and cannot be effectively treated. Understanding the molecular mechanisms underlying liver cancer development is an essential first step in early diagnosis of liver cancer. In this report, we present strong evidence to indicate that the hedgehog pathway is frequently activated in liver cancers. Our data further indicate that induced expression of *Shh* may be the major trigger for activated hedgehog signaling in HCCs. How was *Shh* expression induced in HCC? Our preliminary data indicate that the Shh promoter activity is high in Huh7 cells but low in HepG2 cells (our unpublished observation), suggesting that transcriptional upregulation of the *Shh* gene may be the major mechanism for induced expression of *Shh*.

Since hedgehog signaling is frequently activated in HCCs, markers for hedgehog signaling activation, including *Shh*, *PTCH1* and *Gli1*, may be useful for diagnosis of liver cancers. In most cases, *Gli1* and *PTCH1* were expressed in the tumor,

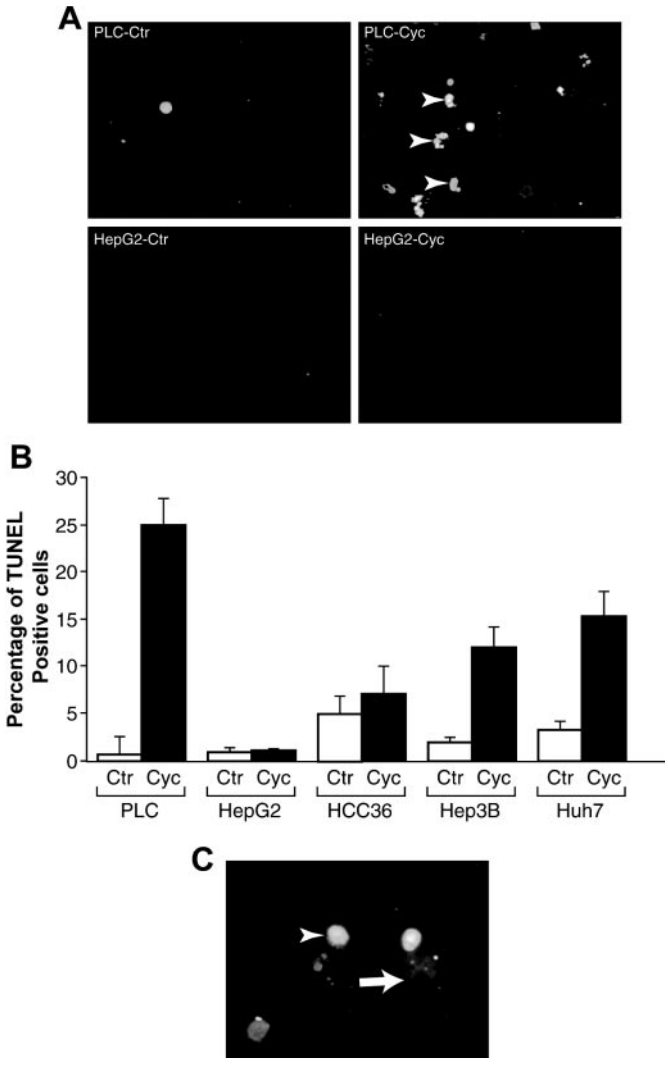

Fig. 4. Targeted inhibition of hedgehog signaling induces apoptosis in hepatocellular carcinoma cells. TUNEL assays (**A**) for detection of apoptosis were measured after treatment with 2 μM KAAD-cyclopamine (Cat# K317000, Toronto Research Chemicals) in PLC (8 h) and in HepG2 (36 h). During treatment, we used 0.5% FBS in DMEM for cell culture. TUNEL positive cells (light grey, indicated by arrowheads) were observed in PLC cells after KAAD-cyclopamine treatment. The percentages of TUNEL positive cells in all five cell lines are quantified in **B.** Please note that different cell lines have different sensitivities to KAAD-cyclopamine. Whereas treatment of 2 μM KAAD-cyclopamine for 8 h was sufficient to induce cell death in PLC cells, a longer treatment was needed for Hep3B (48 h) and Huh7 cells (36 h) (also see Materials and methods for drug treatment). TUNEL positive cells ($n = 500$) were counted under a fluorescent microscope, and the experiment was repeated three times with similar results. TUNEL assays following ectopic Gli1 expression and KAAD-cyclopamine treatment (**C**) demonstrated that cells positive for ectopic Gli1 expression [dark grey staining, detection by c-myc tag as reported previously (27)] were TUNEL negative. Gli1 was expressed in Huh7 cells under the control of the CMV promoter. Transfected cells were treated with 2 μM KAAD-cyclopamine or 2 μM tomatidine in 0.5% FBS in DMEM for 48 h. We found that Gli1-expressing cells ($n = 500$) were all TUNEL negative.

not in the liver tissues adjacent to the tumor. However, in nine cases, we detected expression of *Gli1* and *PTCH1* in both the tumor and the adjacent liver tissues, which were confirmed by real-time PCR in one case (#84) (see Supplementary Table 1 for details). Further analysis indicated that tissue abnormalities were present in these adjacent liver tissues with expression of *Gli1* and *PTCH1*, ranging from small cell dysplasia, dysplastic nodules to microscopic HCCs. In contrast, a non-cancerous liver tissue (as shown in supplementary Figures 2E, 3E and 4E) did not have any detectable expression of *Shh*, *PTCH1* and *Gli1*. Thus, it appears that hedgehog signaling activation occurs in early lesions of HCCs. Further studies of hedgehog signaling in different stages of HCCs, particularly early stages, will establish the basis for early diagnosis of HCC through detection of *Gli1*, *PTCH1* and *Shh*.

Another important pathway involved in HCC is the Wnt pathway via mutations of β-catenin or axin (28–31). We have investigated the association of hedgehog signaling with the Wnt pathway in liver cancer. We detected β-catenin protein localization by immunohistochemistry in tumors with activated hedgehog signaling. Only 1 in 20 tumors with hedgehog signaling activation had nuclear β-catenin, a major indicator for the canonical Wnt signaling, suggesting that hedgehog signaling activation may be a distinct abnormality from β-catenin activation in HCCs.

Therapeutic perspective of liver cancer through targeted inhibition of the hedgehog pathway

Our studies also indicate that targeted inhibition of hedgehog signaling may be effective in treatment of HCCs. We demonstrate in this report that SMO antagonist, KAAD-cyclopamine, or Shh neutralizing antibodies specifically induce apoptosis in HCC cells with activated hedgehog signaling. The hedgehog pathway is not activated in HepG2 cells, and these cells are not sensitive to these reagents. In our studies, variable sensitivities were observed in different cell lines. For PLC cells, treatment with 2 μM KAAD-cyclopamine for 8 h caused apoptosis in many cells. In contrast, a similar rate of cell death was observed in Huh7 cells after treatment (2 μM KAAD-cyclopamine) for 36 h. This difference may be due to other genetic alterations in different cell lines. Further understanding of the molecular basis for cell sensitivity to KAAD-cyclopamine will help us to design better ways to treat HCC in the future. Thus, it may be possible in the future to treat the subsets of liver cancer with hedgehog signaling inhibitors (e.g. KAAD-cyclopamine).

While this manuscript is being reviewed, two other groups have reported similar data on hedgehog signaling in HCCs (32,33).

Supplementary material

Supplementary material is available at: http://www.carcin.oxfordjournals.org/

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Conflict of Interest Statement: None declared.

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