The prognostic significance and therapeutic potential of hedgehog signaling in intrahepatic cholangiocellular carcinoma

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Running Title: prognostic and therapeutic significance of Hh pathway in ICC

Key words: hedgehog signaling; intrahepatic cholangiocellular carcinoma; prognosis; therapeutics; glioma-associated oncogene family zinc finger

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No potential conflicts of interest were disclosed.
Abstract

Purpose: The correlation of the Hh signaling pathway with the progression, prognosis and therapeutics of intrahepatic cholangiocellular carcinoma (ICC) has not been well documented. The study aimed to investigate the expression, prognostic significance and therapeutic value of Hh components in ICC.

Experimental Design: Two independent cohorts of 200 patients with ICC were enrolled. By real-time PCR and immunohistochemistry assay, Hh components expression was evaluated. The prognostic values of Hh proteins were identified and verified. Cyclopamine or siRNA targeting Gli was used to block the Hh signaling. Cell proliferation and apoptosis were observed by CCK8, cell cycle and Annexin V staining assays. In vivo murine tumor model was used to evaluate the role of Hh in ICC.

Results: In ICC tissues, the Gli1 nuclear immune-intensity was associated with intrahepatic metastasis and the expression of Gli2 was associated with intrahepatic metastasis, venous invasion and UICC pT characteristics. In survival analysis, high Gli1 or Gli2 expressers had an unfavorable overall survival (OS) prognosis and a shorter Disease-free survival (DFS) than those with low expression. In multivariate analysis, Gli1 expression was found to be an independent prognostic factor of OS, which was validated by another independent cohort. Further, blocking the Hh signaling by cyclopamine or siRNA targeting Gli1 resulted in apoptosis and growth inhibition in ICC cells.

Conclusions: This study demonstrates, for the first time, activation of Hh pathway associated with the progression and metastasis in ICC, which may provide prognostic and therapeutic values for this tumor.
Translational relevance

This study shows that hedgehog signaling is activated in intrahepatic cholangiocellular carcinoma compared with normal cholangiocytes. Higher Gli1 and Gli2 protein levels in ICC tissues are significantly associated with unfavorable overall survival prognosis and shorter disease-free survival. Gli1 is an independent prognostic factor for clinical outcome in patients with ICC. Blocking the Hh signaling by chemical inhibitor or siRNA targeting Gli1 resulted in apoptosis and growth detention in cultured cells and xenograft tumors. These findings imply that the intratumoral Gli1 levels of ICC can be used to identify subgroups of patients with a favorable or poor overall survival prognosis and Gli1 may have a potential clinical application as a biomarker and a therapeutic target for ICC. Regulation of Hh signaling by agents may influence proliferation and apoptosis of ICC cells during tumor progression that provides an innovative strategy for future clinical trials.
Introduction

Intrahepatic cholangiocellular carcinoma (ICC) is a malignant neoplasm originating from epithelium of the biliary tree with high mortality (1), and a rare primary malignant liver tumor compared with hepatocellular carcinoma (HCC). Unfortunately, the vast majority of patients with cholangiocarcinoma don't have optimal situation for curative surgery when diagnosis is confirmed. Patients with resection generally have the higher recurrence rate (2,3). The conventional chemotherapy and radiation therapy to date have been proved to play limited effective in improving long-term survival and still cannot be satisfied (4). So, the mortality from ICC is very high, with the 5-year survival rates being <15-20% in most series (5, 6). Although several molecules have been reported to be associated with prognosis and metastasis of ICC (7), more valuable biomarkers are needed to predict the clinical outcome or provide therapeutic values of patients with ICC.

Hedgehog (Hh) was first identified in a Drosophila screen for genes important in early embryonic development (8). Sonic (shh), Indian Hh (ihh) and desert Hh (dhh) are three mammalian Hh genes that have been identified (9). Two transmembrane proteins function to transduce the hedgehog signal: Patched (Ptc) and Smoothened (Smo). Smo can transduce signals intracellularly, and this results in the nuclear localization of the transcription factor Glioma-associated oncogene homolog (Gli). Three Gli proteins, Gli1, Gli2 and Gli3 are known to be present in vertebrates. The three Gli proteins act in a combinatorial fashion, with Gli1 and Gli2 being the major positive intermediaries of Hh signaling. Hedgehog interacting protein (Hhip) was
identified as a protein that can bind to Shh and inhibit hedgehog signaling (10). Recent evidence has demonstrated that Hh plays an important role in multiple tumor types, for example, basal cell carcinoma (11,12), small cell lung cancer (13), prostate cancer (14), gastric cancer (15), esophageal cancer (16), pancreatic cancer (15,17), and HCC (18,19). The reports have been shown that dysregulation of hedgehog signaling may contribute to tumorigenesis.

Although limited studies have reported the role of Hh in cholangiocellular carcinoma, the correlation of the Hh signaling pathway with the prognosis and therapeutics of ICC has not been well documented. In the current study, expression of the Hh signaling pathway in ICC was detected and the relationship between Hh proteins and clinicopathologic characteristics was investigated. In addition, the response of ICC cells to inhibition of the hedgehog signaling by cyclopamine or siRNA was assessed by evaluating cell proliferation, apoptosis and tumor growth.

Materials and Methods

Patients and tissue samples

Formalin-fixed and paraffin-embedded ICC tissues from 200 consecutive patients and normal liver tissues from 8 patients who underwent primary hepatectomy due to ICC or metastatic liver tumors in our Hospital from January 2002 to December 2004 were retrieved for immunohistochemistry. We divided the patients into training set (all the 108 case of patients in 2002) and validation cohort (the 92 case of patients underwent hepatectomy in 2003-2004). The study population consisted of patients with ICC as
confirmed by pathological analysis. Tumor differentiation was defined according to the Edmondson grading system. Tumor staging was determined according to the sixth edition of the tumor-node-metastasis (TNM) classification of the International Union Against Cancer. The selection of this specific material was performed to include patients who underwent surgery alone without chemotherapy or radiotherapy at a time when these adjunctive therapies were not the standard of treatment. In this way, the analysis of data in this series will reflect the actual impact of the tumour biology on the clinical outcome. The patients included in this series also had: available paraffin-embedded tumor tissue; the tumor cell subtype proven by immunohistochemistry. Patients were excluded from this cohort with the following exclusion criteria: uncontrolled infection; previously received any anticancer therapy; pregnancy and lactation; prior malignancy; impaired heart, lung, liver, or kidney function; previous malignant disease. The follow-up period was defined as the interval from the date of operation to the date of death or the last follow-up. Deaths from other causes were treated as censored cases. All patients were observed until December 2010, ranged from 2 to 82 months (median, 17 months).

Fresh-frozen ICC tissues from 41 patients and normal intrahepatic bile duct epithelia from 5 patients who underwent primary hepatectomy between 2001 and 2007 were used for RNA extraction. Overall survival (OS) was defined as the interval between the dates of surgery and death. Disease-free survival (DFS) was defined as the interval between the dates of surgery and recurrence; if recurrence was not diagnosed, patients were censored on the date of death or the last follow-up. Informed
consent was obtained from all patients before subsequent use of their resected tissues. The present study was performed in accordance with the ethical standards of the Helsinki Declaration in 1975, after approval of the Ethics Committee of Human Experimentation of Second Military Medical University.

**Tissue microarray and immunohistochemistry**

Tissue microarray (TMA) slides were prescreened with hematoxylin and eosin-staining (Shanghai Biochip Company, Ltd., Shanghai, China). Two cores were taken from each formalin-fixed, paraffin-embedded ICC samples and normal liver samples by using punch cores that measured 0.8 mm in greatest dimension from the center of tumor foci. The sections were heated in a primary polyclonal antibodies against Shh (sc-9024), Gli1 (sc-20687) (Santa Cruz Biotechnology, Santa Cruz, CA) and Gli2 (ab-26056) (Abcam Ltd., Cambridge, United Kingdom) at 1:100 dilution. Finally, the visualization signal was developed with diaminobenzidine and the slides were counterstained in hematoxylin. Stained sections were evaluated in a blinded manner without prior knowledge of the clinical information using the German immunoreactive score, immunoreactive score (IRS). Briefly, the IRS assigns sub-scores for immunoreactive distribution (0–4) and intensity (0–3), then multiplies them to yield the IRS score. The percent positivity was scored as “0” (<5%), “1” (5–25%), “2” (25–50%), “3” (50–75%) or “4” (>75%). The staining intensity was score as “0” (no staining), “1” (weakly stained), “2” (moderately stained) or “3” (strongly stained). Cases with discrepancies in IRS score were discussed together with
other pathologists until consensus was reached. Nuclear staining is considered as active form of Glis, IRS score was used to assess nuclear immune-reactivity of Glis and the patients were separated into high and low expression groups.

**Cell culture and chemicals**

The intrahepatic cholangiocarcinoma cell line RBE and HCCC9810 was purchased from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). The cholangiocarcinoma cell lines QBC939 was purchased from Xiangya Central Experiment Laboratory (Hunan, China). All cells were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic solution (Sigma-Aldrich, St. Louis, MO), and maintained at 37°C in an atmosphere of humidified air containing 5% CO₂. A specific hedgehog signal inhibitor, KAAD-cyclopamine, was purchased from Toronto Research Chemicals (North York, Canada) and its nonfunctional analogue, tomatidine hydrochloride, was purchased from Sigma-Aldrich (St. Louis, MO).

**RNA collection, cDNA synthesis, and real-time PCR analysis**

Pure normal intrahepatic cholangiocytes from human liver tissues were isolated by laser capture microdissection (LCM) as described previously (20). Total RNA was extracted from fresh-frozen tumor specimens, healthy control tissues, and cell lines in Trizol (Invitrogen, Carlsbad, CA). Reverse transcription of total RNA was performed using random hexamers (Roche Diagnostics, Penzberg, Germany) and SuperScriptII
reverse transcriptase (Invitrogen). Real-time PCR of the respective genes was carried out with 40 ng complementary DNA, 500 nM forward and reverse primer, and iTaqSYBRGreen Supermix (Bio-Rad Laboratories, Hercules, CA) in a final volume of 20 μl.

**Cell proliferation assay and apoptosis analysis**

Cholangiocarcinoma cells (5 × 10^3 cells per well) were seeded in 96-well plates and cultured overnight at 37°C, then the cells were treated with different concentrations of KAAD-cyclopamine or tomatidine for another 48 hours or were transfected for indicated time. Cell proliferation was detected by CCK-8 assay at various time points according to the guidance of the manufacturer. Apoptosis was also assessed by using an annexinV-FITC apoptosis detection kit (Invitrogen, V13241) according to the manufacturer’s protocol. For clonogenic assays as described (21), different cell lines were seeded in 6-well plates (5000 cells/well) and grown under the indicated conditions for 10 days. The number of colonies (defined as cell clusters consisting of at least 50 cells) was quantified by Analysis software.

**Statistical analysis**

The Pearson χ² test or Fisher’s exact test was used to analyze the relationship between Gli1 or Gli2 expression and the clinicopathologic features. Survival curves were calculated using the Kaplan-Meier method and compared by the log-rank test. The Cox proportional-hazard regression model was used to explore the effects of the clinicopathological variables and Gli1 and Gli2 expression on survival. SPSS 15.0
software (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses and a p value of less than 0.05 was considered significant.

Results

Hedgehog signaling components mRNA expression in intrahepatic cholangiocellular carcinoma specimens

RNA extracted from 41 cases of ICC specimens and 5 normal cholangiocytes tissues showed transcripts of all essential hedgehog signaling components, including genes encoding the extracellular ligands Shh and Ihh, the transmembrane protein Smo, and the known hedgehog target genes Gli1, Gli2, Gli3 and Ptch1. The hedgehog pathway competing component Hhip1 was also included. As the results shown in Figure 1, Ihh, Shh, Ptch1, Smo, Gli1 and Gli2 mRNA expressions were markedly increased in 78% (32 of 41), 87.8% (36 of 41), 89.2 % (34 of 41), 85.4% (35 of 41), 65.9% (27 of 41), and 87.8% (36 of 41), respectively. While Gli3 and Hhip1, the two known negative factors in hedgehog signaling, showed only 36.6% (15 of 41) and 17.1 (7 of 41) samples high expression. The Hh target genes, c-Myc and Bcl-2 were also detected and further confirmed activation of the signaling (Supplement fig. 1). For further validation of the results, we applied LCM to obtain tumor tissues from another 30 ICC samples to evaluate Hh components expression and found the similar expression pattern (Supplement fig. 2A). We detected some key genes' copy number in fresh ICC tissues and found there few copy number changes in these genes (Supplement fig. 2B).
Immunohistochemical study of Shh, Gli1, and Gli2 in intrahepatic cholangiocellular carcinoma and normal liver tissues

Expression of Shh, Gli1, and Gli2 proteins was determined by immunohistochemistry staining in ICC cancer tissues and 8 cases of normal liver tissues (Supplement fig. 3). Shh was mainly localized in the cytoplasm of the cancer cells. Of the training cohort patients, 93.5% (101 of 108) were classified as Shh positive. There were 87.9% (95 of 108) specimens with positive Gli1 expression, and the immunostaining was distributed in cytoplasm and nuclear in these specimens. There were 92.6% (100 of 108) specimens with positive Gli2 expression, and the positive lesion was also in the cytoplasm and nuclear. Shh, Gli1 and Gli2 were present negative or weak intensity in the bile duct of normal liver tissues and this intensity was considered as normal-like for the subsequent scoring of cancer areas. Cholangiocarcinoma-specific staining, CK-19 was also performed on the tumor samples to confirm positive staining cells were ICC cells (Supplement fig. 3G, H).

Association of Shh, Gli1, and Gli2 expression with the clinicopathological features

We analyzed the relationship between Shh, Gli1, Gli2 protein expression and clinical features of ICC in high and low expression groups based on the immunohistochemistry analysis. We found significant correlation between Gli1 expression and clinical features. As shown in Table 1 (training cohort), Gli1 protein level was associated with intrahepatic metastasis clinical feature (p=0.021). In all, 17 (30.9%) patients without intrahepatic metastasis had high Gli1 expression compared
with 28 (52.8%) of positive intrahepatic metastasis patients. The expression of Gli2 was also associated with intrahepatic metastasis (p=0.032). In addition, Gli2 expression significantly correlated with venous invasion (p=0.032) and UICC pT clinical characteristics (p=0.014). However, Shh did not correlate with any of the clinical characteristic. The similar results were confirmed in the validation cohort (Supplement Table 1).

**Significant prognostic values of Gli1 and Gli2 expression for patients with intrahepatic cholangiocellular carcinoma**

To investigate the relationship between Shh, Gli1, Gli2 expression and clinical prognosis, the ICC patients were followed up. The 1-year, 3-year, and 5-year OS were 65.7%, 29.6% and 14.8% for all of the patients in this study. Based on the each protein intratumoral densities, the patients were divided into two groups: high intratumoral protein density (IRS over 6, as described in Materials and methods) and low group (IRS 0-6). Using Kaplan-Meier analysis we found that "high Gli1 expressers" had an unfavorable overall survival prognosis and a shorter DFS than those with low Gli1 expression (training set Fig. 2A, 2B; validation cohort Fig. 2E, 2F). The 1-year, 3-year, and 5-year OS were 68.3%, 41.3% and 20.6% for low Gli1 expression patients in training cohort. While the high Gli1 expressers’ 1-year, 3-year, and 5-year OS were 48.9%, 13.3% and 11.1%, respectively. Turning to the Gli2, patients with high reactivity of Gli2 have poor prognosis compared with those with low reactivity for either OS or DFS (training set Fig. 2C, 2D; validation cohort Fig. 2G, 2H). The 1-year, 3-year, and 5-year OS were 67.6%, 39.7% and 22.1% for low
Gli2 expression patients in training set. While the high Gli2 expressers’ 1-year, 3-year, and 5-year OS were 47.5%, 12.5% and 7.5%, respectively.

Univariate analyses of clinical variables considered as potential predictors of survival are shown in Table 2 and Supplement Table 2. By Cox regression analyses, the parameters gender, serum carcinoembryonic antigen (CEA), tumor size, intrahepatic metastasis, lymph node metastasis, venous invasion, international union against cancer T classification (UICC pT), and UICC stage were identified as potential predictors of DFS and OS. In this model, Gli1 and Gli2 also showed correlating with DFS and OS. As Gli1 and Gli2 showed association with histopathological variables known to affect prognosis, a multivariate analysis was performed to assess the independence of Gli1 and Gli2 prognosis. It was shown that Gli1, together with those of tumor size, intrahepatic metastasis, lymph node metastasis, was strongly associated with OS, but Gli1 was not significantly correlated with DFS. The results indicated that Gli2 may be not an independent prognostic factor for both DFS and OS.

**Lentivirus and adenovirus-delivered Gli1 siRNA suppressed cholangiocarcinoma cells growth in vitro and in vivo**

To validate the clinical significance of Gli1, we constructed the lentivirus and adenovirus-delivered siRNA (Lv-SiRNA, AdSiRNA) targeting Gli1 or Gli2. The Lv-SiRNA significantly suppressed Gli1 and Gli2 expression levels (Supplement fig. 4A, 4B). Further, Proliferation of Gli1-siRNA cells was evidently slowed down in RBE, HCCC9810 and QBC939 cells (Fig. 3A, Supplement fig.4E). As Figure 3B
showed, depleting Gli1 markedly reduced colony formation of RBE, HCCC9810 and QBC939. Stably transfected Gli1-overexpressed RBE cell was constructed and subcutaneously injected into flanks of NOD-SCID mice. Forced expression of Gli1 enhanced tumor formation in vivo, while the control cell did not grow into tumor (Fig.3C). In addition, suppressing expression of Gli1 in QBC939 cells significantly inhibited tumor growth, but interference of Gli2 didn't show effects (Supplement fig. 4F). Further, we found in Gli1-siRNA RBE cells, distinct G2/M phase arrest was observed (Fig. 3D), while Lv-SiRNA targeting Gli2 didn't show effects. Then, we detected the apoptosis in Lv-SiRNA treated cells, and found inhibition of Gli1 markedly induced cell apoptosis, while suppression of Gli2 had not effect (Fig. 3E).

In cholangiocarcinoma cells, the AdSiGli1 significantly decreased the expression of Gli1 mRNA and protein (Supplement fig. 4C, 4D). Knocking down Gli1 caused RBE, HCCC9810 and QBC939 cells growing slowly compared with cells with normal expression of Gli1 (Fig. 4A). AdSiGli1 also significantly inhibited colony formation of RBE and HCCC9810 cells (Fig.4B). We tested whether blocking the Shh-Gli signaling pathway with cyclopamine, a selective Smo inhibitor, could affect the proliferation and survival of ICC cells. The results showed that cell proliferation was significantly reduced by the inhibitor (Fig. 4C) and apoptosis was induced at a late stage and at an early stage (Fig.4D, 4E). We further tested the role of hedgehog pathway activity in vivo by treating established subcutaneous xenograft tumors in nude mice with daily subcutaneous injections of cyclopamine or DMSO. After treatment with cyclopamine, the tumor growth was inhibited obviously (Supplement
fig.4G).

**Discussion**

ICC is an uncommon malignant disease with inadequate therapeutic methods, studies of the underlying molecular mechanism will provide potential therapeutic target. Recent years, the prognostic value of Hh components in some tumor types was reported (22-26). In the present study, we clearly demonstrate that many genes of hedgehog signaling up-regulated in clinical ICC samples. The expression patterns of Hh pathway genes may be associated with bile duct epithelium tumorigenesis. A significant association was found between Gli1 and intrahepatic metastasis feature. Meanwhile, Gli2 staining associated with intrahepatic metastasis, venous invasion and UICC T factor classification. Compared with Gli2, Gli1 was an independent prognostic predictor for overall survival.

To the best of our knowledge, there was no report on the correlation of the Hh signaling pathway with the progression and prognosis of ICC. In other type of tumors, most researches focused on Gli1, but not Gli2 for prognosis study. It is reported that Gli1 was an indicator for a poor prognosis in patients with colon cancer (25) and with head and neck squamous cell carcinoma (26). Zhu W et al found that esophageal squamous cell carcinoma patients with high Gli1 expression have the shorter survival time (22). Gli1 expression is an independent prognostic marker of ovarian cancers (27). Turning to Gli2, although no report about prognostic correlation of Gli2 and tumors, there were some functional studies of the protein in tumor cells growth and
tumorigenicity (28-30). Among the Gli members, Gli1 possesses only an activator domain, whereas Gli2 contains both activator and repressor domains, and Gli3 mostly functions as a repressor (31). 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 that Gli1 can have a preference for different targets in different cancer types (32, 33). Indeed, Gli1 itself is a transcriptional target of the Hh pathway, Gli1 expression serves as a reliable indicator of activated Hh signaling, and elevated Gli1 expression was linked with cancer development and progression (14).

Our results show that cyclopamine could affect the proliferation and growth of ICC cells. A recent report stated that cyclopamine and 5E1 treatments effectively inhibited cell proliferation, migration and invasion of extra-hepatic cholangiocarcinoma cells by down-regulating Gli1 and Gli2, which provided further evidences to confirm the important role of hedgehog-Gli1 in cholangiocarcinoma (34). Given that some tumor cells were not sensitive or had no response to cyclopamine (35), we used siRNA targeting Gli1. The fact that inhibition of hedgehog signaling leads to a dramatic decrease in ICC cells growth in vitro and in vivo by a massive induction of apoptosis indicates an important role of a targeted hedgehog signaling blockade for future therapeutic interventions in this tumor. In addition, some studies revealed that Hh signaling in development was typically mediated through paracrine effects. Yauch et al reported that Hh-Gli activation was required in the tissue mesenchyme surrounding
pancreatic cancer cells to support tumor growth by paracrine effects (35). Gores' group reported an interesting work which revealed that myofibroblast derived PDGF promoting survival of cholangiocarcinoma cells (36). Therefore, the role of Hh paracrine signaling may be required for the progression of ICC, which needs to be further investigated.

Taken together, our study demonstrated that overexpressed Gli1 and Gli2 were correlated with progression and metastasis in ICC. Gli1 is considered to be an independent prognostic factor for overall survival in patients with ICC. Elucidating Hh pathway in cancer cells and careful classification of patients may help for development of novel therapeutic strategies.

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**References**

Figure legends

Figure 1. mRNA expression of Hedgehog signaling genes in intrahepatic cholangiocellular carcinoma specimens. *Ihh, Shh, Ptc1, Smo, Gli1, Gli2, Hhip* and *Gli3* mRNA expression levels were detected in 41 clinical ICC specimens and 5 cases normal bile vessel tissue obtained by laser capture microdissection. The value represented Log2 of qRT-PCR value of ICC tumor to the average mRNA of five normal bile ducts. The light grey samples (>0) showed that the mRNA levels of the ICC tissues were higher than the average of normal bile ducts.

Figure 2. Kaplan-Meier curves for time to recurrence and overall survival of patients with high or low intratumoral Gli1 or Gli2 features in training cohort (A-D) and validation cohort (E-H). The different subgroups were plotted according to the cut-off value of Gli1 or Gli2 level defined as the median of the cohort.

Figure 3. Lentivirus-delivered Gli1-siRNA induced cholangiocarcinoma cells growth arrest and apoptosis. (A) Lv-Gli1-siRNA infection suppressed cholangiocarcinoma cells proliferation. RBE and HCCC9810 cells were infected with lentivirus (20 MOI) targeting Gli1 or Gli2, then the cell proliferation were assayed by CCK8. (B) Clonogenic assay of three cholangiocarcinoma cell lines after Gli1 or Gli2 knockdown with lentivirus-delivered siRNA. (C) Overexpression of Gli1 enhanced RBE cell growth in NOD-SCID mice. The vector control (VT) cells and tumor tissues of Gli1-overexpression (OV) cells were lysated and subjected to western blot assay. (D) Deletion of Gli1 expression by siRNA induced G2/M phase arrest of RBE cells. (E) Inhibition of Gli1 expression induced apoptosis in RBE cells.
Figure 4. Adenovirus-delivered Gli1 siRNA and cyclopamine suppressed cholangiocarcinoma cells growth. (A) AdSi-Gli1 suppressed cholangiocarcinoma cells proliferation in vitro. (B) Clonogenic assay of RBE and HCCC cell lines after Gli1 knockdown with adenovirus-delivered siRNA. (C) Cyclopamine treatment effects on proliferation of cholangiocarcinoma cells. RBE and HCCC9810 were treated with KAAD-cyclopamine or the inactive analogue tomatidine with 0-20 M for 48 hours and the cell proliferation was detected by CCK-8 assay. (D, E) Cyclopamine treatment effects on proliferation of cholangiocarcinoma cells. Cells were treated for 24 and 48 hours with 10μM cyclopamine or tomatidine followed by apoptosis assay using FITC-conjugated Annexin \( \square \) and propidium iodide (PI).
Figure 1

[Graph showing the expression levels of various genes (Hh1, Shh, Ptc1, Smo, Gl1, Gl2,Gl3, Hhip) in tumor and normal tissues on a logarithmic scale.]
Figure 2

A

Overall survival rate (%)

Gli1 high
Gli1 low
p = 0.0038

Disease-free survival rate (%)

Gli1 high
Gli1 low
p = 0.0319

Months after operation

B

Overall survival rate (%)

Gli2 high
Gli2 low
p = 0.0011

Disease-Free Survival Rate(%)

Gli2 high
Gli2 low
p = 0.0164

Months after operation

C

Overall survival rate (%)

Gli1 high
Gli1 low
p = 0.000

Disease-Free Survival Rate(%)

Gli1 high
Gli1 low
p = 0.0047

Months after operation

D

Overall survival rate (%)

Gli2 high
Gli2 low
p = 0.0139

Disease-Free Survival Rate(%)

Gli2 high
Gli2 low
p = 0.0145

Months after operation
Figure 3

A

RBE

proliferation rate

HCCC9810

proliferation rate

B

GFP   siGli1   siGli2

RBE

HCCC9810

QBC939

C

RBE

VT

OV

Myc-tag Gli1

GAPDH

D

Percentage (%)

RBE

Con-siRNA  Gli2-siRNA  Gli1-siRNA

G0/G1

S

G2/M

E

Apoptosis (%)

RBE

Con-siRNA  Gli2-siRNA  Gli1-siRNA
**Figure 4**

A

- **RBE**
  - Proliferation rate over time (0, 24, 48, 72 hours)
  - Graph showing proliferation rates for RBE cells.
  - Key indicating significance levels.

- **HCCC9810**
  - Proliferation rate over time (0, 24, 48, 72 hours)
  - Graph showing proliferation rates for HCCC9810 cells.
  - Key indicating significance levels.

- **QBC939**
  - Proliferation rate over time (0, 24, 48, 72 hours)
  - Graph showing proliferation rates for QBC939 cells.
  - Key indicating significance levels.

B

- **Mock**
  - Images of cells

- **Adsi-Gli1**
  - Images of cells

- **Adsi-blank**
  - Images of cells

B

- **RBE**
  - Images of cells

- **HCCC**
  - Images of cells

C

- **RBE**
  - Graph showing effects of KAAD-cyclopamine and Tomatidine.
  - Key indicating significance levels.

- **HCCC9810**
  - Graph showing effects of KAAD-cyclopamine and Tomatidine.
  - Key indicating significance levels.

D

- **Mock**
  - Images of cells

- **Tomatidine**
  - Images of cells

- **KAAD-cyc**
  - Images of cells

D

- **RBE**
  - Images of cells

- **HCCC**
  - Images of cells

- **QBC**
  - Images of cells

E

- **HCCC9810**
  - Bar graph showing percentage of apoptosis cells (Annexin positive).
  - Key indicating significance levels.

- **RBE**
  - Bar graph showing percentage of apoptosis cells (Annexin positive).
  - Key indicating significance levels.

- **QBC939**
  - Bar graph showing percentage of apoptosis cells (Annexin positive).
  - Key indicating significance levels.
Table 1 Relationship between Gli1, Gli2 protein expression and clinicopathologic characteristics in training set (n=108)

<table>
<thead>
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<th>Characteristics</th>
<th>No. patients (%)</th>
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<th>P value</th>
<th>Gli2 immunoreactivity</th>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>44 (40.7)</td>
<td>23 21</td>
<td>0.289</td>
<td>25 19</td>
<td>0.273</td>
</tr>
<tr>
<td>Positive</td>
<td>64 (59.3)</td>
<td>40 24</td>
<td></td>
<td>43 21</td>
<td></td>
</tr>
<tr>
<td>Serum CA19-9 (units/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤37</td>
<td>55 (50.9)</td>
<td>35 20</td>
<td>0.255</td>
<td>37 18</td>
<td>0.345</td>
</tr>
<tr>
<td>&gt; 37</td>
<td>53 (49.1)</td>
<td>28 25</td>
<td></td>
<td>31 22</td>
<td></td>
</tr>
<tr>
<td>Largest tumor size (cm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤5</td>
<td>34 (31.5)</td>
<td>20 14</td>
<td>0.944</td>
<td>24 10</td>
<td>0.266</td>
</tr>
<tr>
<td>&gt; 5</td>
<td>74 (68.5)</td>
<td>43 31</td>
<td></td>
<td>44 30</td>
<td></td>
</tr>
<tr>
<td>Intrahepatic metastasis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>55 (50.9)</td>
<td>38 17</td>
<td>0.021</td>
<td>40 15</td>
<td>0.032</td>
</tr>
<tr>
<td>Positive</td>
<td>53 (49.1)</td>
<td>25 28</td>
<td></td>
<td>28 25</td>
<td></td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>76 (70.0)</td>
<td>46 30</td>
<td>0.476</td>
<td>48 28</td>
<td>0.948</td>
</tr>
<tr>
<td>Positive</td>
<td>32 (30.0)</td>
<td>17 15</td>
<td></td>
<td>20 12</td>
<td></td>
</tr>
<tr>
<td>Venous invasion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>66 (61.1)</td>
<td>39 27</td>
<td>0.841</td>
<td>48 18</td>
<td>0.008</td>
</tr>
<tr>
<td>Positive</td>
<td>42 (38.9)</td>
<td>24 18</td>
<td></td>
<td>20 22</td>
<td></td>
</tr>
<tr>
<td>Perineural invasion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>94 (87.0)</td>
<td>54 40</td>
<td>0.628</td>
<td>58 36</td>
<td>0.482</td>
</tr>
<tr>
<td>Positive</td>
<td>14 (13.0)</td>
<td>9 5</td>
<td></td>
<td>10 4</td>
<td></td>
</tr>
<tr>
<td>Histological grading</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well</td>
<td>3 (2.8)</td>
<td>2 1</td>
<td>0.899</td>
<td>2 1</td>
<td>0.885</td>
</tr>
<tr>
<td>Moderate</td>
<td>57 (52.7)</td>
<td>34 23</td>
<td></td>
<td>37 20</td>
<td></td>
</tr>
<tr>
<td>Poorly</td>
<td>48 (44.4)</td>
<td>27 21</td>
<td></td>
<td>29 19</td>
<td></td>
</tr>
<tr>
<td>UICC pT</td>
<td>0.180</td>
<td>0.014</td>
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<td>--------</td>
<td>-------</td>
<td>-------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1+2</td>
<td>49 (45.4)</td>
<td>32</td>
<td>17</td>
<td>37</td>
<td>12</td>
</tr>
<tr>
<td>3+4</td>
<td>59 (54.6)</td>
<td>31</td>
<td>28</td>
<td>31</td>
<td>28</td>
</tr>
<tr>
<td>UICC stage</td>
<td>0.402</td>
<td>0.086</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I+II</td>
<td>41 (38.0)</td>
<td>26</td>
<td>15</td>
<td>30</td>
<td>11</td>
</tr>
<tr>
<td>III+IV</td>
<td>67 (62.0)</td>
<td>37</td>
<td>30</td>
<td>38</td>
<td>29</td>
</tr>
<tr>
<td>Resection status</td>
<td>0.888</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R0</td>
<td>93 (86.1)</td>
<td>54</td>
<td>39</td>
<td>49</td>
<td>44</td>
</tr>
<tr>
<td>R1/R2</td>
<td>15 (13.9)</td>
<td>9</td>
<td>6</td>
<td>8</td>
<td>7</td>
</tr>
</tbody>
</table>
Table 2 Univariate and multivariate Cox regression analyses GlI1, Gli2, Shh for DFS or OS of patients in the training cohort (n=108).

<table>
<thead>
<tr>
<th>Variables</th>
<th>DFS Hazard ratio (95% CI)*</th>
<th>p Value</th>
<th>OS Hazard ratio (95% CI)*</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Univariate analysis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gli 1 (high vs low)</td>
<td>1.614 (1.063-2.452)</td>
<td>0.025</td>
<td>1.808 (1.193-2.738)</td>
<td>0.005</td>
</tr>
<tr>
<td>Gli 2 (high vs low)</td>
<td>1.660 (1.085-2.540)</td>
<td>0.020</td>
<td>1.978 (1.293-3.028)</td>
<td>0.002</td>
</tr>
<tr>
<td>Shh (high vs low)</td>
<td>0.686 (0.456-1.033)</td>
<td>0.071</td>
<td>0.683 (0.454-1.027)</td>
<td>0.067</td>
</tr>
<tr>
<td>Age (&gt;60 years vs ≤60 years)</td>
<td>1.280 (0.819-2.000)</td>
<td>0.278</td>
<td>1.211 (0.777-1.886)</td>
<td>0.398</td>
</tr>
<tr>
<td>Gender (male vs female)</td>
<td>0.624 (0.391-0.996)</td>
<td>0.048</td>
<td>0.620 (0.388-0.991)</td>
<td>0.046</td>
</tr>
<tr>
<td>HBV (negative vs positive)</td>
<td>1.399 (0.916-2.135)</td>
<td>0.120</td>
<td>1.254 (0.822-1.913)</td>
<td>0.293</td>
</tr>
<tr>
<td>Serum CA19-9 (&gt;37 units/ml vs ≤37 units/ml)</td>
<td>1.494 (0.989-2.258)</td>
<td>0.057</td>
<td>1.478 (0.983-2.224)</td>
<td>0.061</td>
</tr>
<tr>
<td>Serum CEA (&gt;10 ng/ml vs ≤10 ng/ml)</td>
<td>2.276 (1.165-4.446)</td>
<td>0.016</td>
<td>4.259 (2.125-8.533)</td>
<td>0.000</td>
</tr>
<tr>
<td>Serum AFP (&gt;20 ng/ml vs ≤20 ng/ml)</td>
<td>1.509 (0.912-2.497)</td>
<td>0.110</td>
<td>1.418 (0.860-2.337)</td>
<td>0.171</td>
</tr>
<tr>
<td>Largest tumor size (&gt;5 cm vs ≤5 cm)</td>
<td>2.461 (1.531-3.955)</td>
<td>0.000</td>
<td>2.461 (1.535-3.946)</td>
<td>0.000</td>
</tr>
<tr>
<td>Intrahepatic metastasis (negative vs positive)</td>
<td>2.393 (1.565-3.659)</td>
<td>0.000</td>
<td>2.507 (1.653-3.802)</td>
<td>0.000</td>
</tr>
<tr>
<td>Lymph node metastasis (negative vs positive)</td>
<td>2.467 (1.564-3.891)</td>
<td>0.000</td>
<td>3.385 (2.142-5.349)</td>
<td>0.000</td>
</tr>
<tr>
<td>Venous invasion (negative vs positive)</td>
<td>1.550 (1.015-2.367)</td>
<td>0.043</td>
<td>1.541 (1.013-2.345)</td>
<td>0.043</td>
</tr>
<tr>
<td>Perineural invasion (negative vs positive)</td>
<td>1.317 (0.731-2.372)</td>
<td>0.360</td>
<td>1.287 (0.715-2.318)</td>
<td>0.401</td>
</tr>
<tr>
<td>Histological grading (well vs moderate/ poorly)</td>
<td>0.920 (0.290-2.915)</td>
<td>0.887</td>
<td>0.917 (0.289-2.907)</td>
<td>0.883</td>
</tr>
<tr>
<td>UICC pT (1+2 vs 3+4)</td>
<td>2.813 (1.817-4.353)</td>
<td>0.000</td>
<td>3.080 (1.998-4.748)</td>
<td>0.000</td>
</tr>
<tr>
<td>UICC stage (I+II vs III+IV)</td>
<td>2.898 (1.842-4.558)</td>
<td>0.000</td>
<td>3.268 (2.081-5.132)</td>
<td>0.000</td>
</tr>
<tr>
<td>Resection status (R0 vs R1/R2)</td>
<td>1.399 (0.775-2.524)</td>
<td>0.265</td>
<td>1.596 (0.885-2.878)</td>
<td>0.120</td>
</tr>
<tr>
<td><strong>Multivariate analysis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gli 1 (high vs low)</td>
<td>NA</td>
<td></td>
<td>1.966 (1.283-3.012)</td>
<td>0.002</td>
</tr>
<tr>
<td>Gli 2 (high vs low)</td>
<td>NA</td>
<td></td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Gender (male vs female)</td>
<td>NA</td>
<td></td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Serum CEA (&gt;10 ng/ml vs ≤10 ng/ml)</td>
<td>NA</td>
<td></td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Largest tumor size (&gt;5 cm vs ≤5 cm)</td>
<td>2.334 (1.439-3.785)</td>
<td>0.001</td>
<td>2.484 (1.539-4.010)</td>
<td>0.000</td>
</tr>
<tr>
<td>Intrahepatic metastasis (negative vs positive)</td>
<td>2.416 (1.563-3.733)</td>
<td>0.000</td>
<td>2.459 (1.599-3.781)</td>
<td>0.000</td>
</tr>
<tr>
<td>Lymph node metastasis (negative vs positive)</td>
<td>2.203 (1.379-3.520)</td>
<td>0.001</td>
<td>3.531 (2.170-5.744)</td>
<td>0.000</td>
</tr>
<tr>
<td>Venous invasion (negative vs positive)</td>
<td>NA</td>
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<td>NA</td>
<td></td>
</tr>
<tr>
<td>Perineural invasion (negative vs positive)</td>
<td>NA</td>
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<tr>
<td>UICC pT (1+2 vs 3+4)</td>
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<tr>
<td>UICC stage (I+II vs III+IV)</td>
<td>NA</td>
<td></td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>
*For Gli1, Gli2, Shh, median values were used as the cut-off point for definition of subgroups (low expression and high expression groups).

Univariate analysis, Cox proportional hazards regression; Multivariate analysis, Cox proportional hazards regression; Variables were adopted in multivariate analysis for their prognostic significance by univariate analysis.
The prognostic significance and therapeutic potential of hedgehog signaling in intrahepatic cholangiocellular carcinoma

Liang Tang, Ye-xiong Tan, Bei-ge Jiang, et al.

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