The Prognostic Significance and Therapeutic Potential of Hedgehog Signaling in Intrahepatic Cholangiocellular Carcinoma

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Abstract

**Purpose:** The correlation of the hedgehog 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 hedgehog components in ICC.

**Experimental Design:** Two independent cohorts of 200 patients with ICC were enrolled. By real-time PCR and immunohistochemistry assay, hedgehog components expression was evaluated. The prognostic values of hedgehog proteins were identified and verified. Cyclopamine or siRNA-targeting Gli1 was used to block the hedgehog 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 hedgehog 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 Unio Internationale Contra Cancrum (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. Furthermore, blocking the hedgehog signaling by cyclopamine or siRNA-targeting Gli1 resulted in apoptosis and growth inhibition in ICC cells.

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

*Clin Cancer Res*; 19(8); 2014–24. ©2013 AACR.

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 do not 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 radiotherapy to date have proved to play limited effect in improving long-term survival and it still cannot be satisfied (4). So, the mortality from ICC is very high, with the 5-year survival rates being less than 15% to 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 was first identified in a *Drosophila* screen for genes important in early embryonic development (8). Sonic hedgehog (*Shh*), Indian hedgehog (*Ihh*), and desert hedgehog (*Dhh*) are 3 mammalian hedgehog genes that have been identified (9). Two transmembrane proteins function to transduce the hedgehog signal: patched (Ptch) 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...
Tissue microarray and immunohistochemistry

Tissue microarray slides were prescreened with hematoxylin and eosin staining (Shanghai Biochip Company, Ltd.). 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), and Gli2 (ab-26056; Abcam Ltd.) 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 subscres for immunoreactive distribution (0–4) and intensity (0–3), then multiplies them to yield the IRS score. The percentage positivity was scored as “0” (<5%), “1” (5%–25%), “2” (25%–50%), “3” (50%–75%), or “4” (>75%).
The staining intensity was scored 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 Gli1. IRS score was used to assess nuclear immuno-reactivity of Gli1 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% FBS and 1% antibiotic/antimycotic solution (Sigma-Aldrich) 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 and its nonfunctional analog, tomatidine hydrochloride, was purchased from Sigma-Aldrich.

**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). Reverse transcription of total RNA was conducted using random hexamers (Roche Diagnostics) and SuperScriptII reverse transcriptase (Invitrogen). Real-time PCR of the respective genes was conducted with 40 ng complementary DNA, 500 nmol/L forward and reverse primer, and iTaqSYBRGreen Supermix (Bio-Rad Laboratories) in a final volume of 20 μL.

**Cell proliferation assay and apoptosis analysis**

Cholangiocarcinoma cells (5 × 10^4 cells/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 annexin V–fluorescein isothiocyanate (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 (5,000 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 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 clinicopathologic variables and Gli1 and Gli2 expression on survival. SPSS 15.0 software (SPSS Inc.) 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 Ptc1. The hedgehog pathway competing component Hhip1 was also included. As the results shown in Fig. 1, Ihh, Shh, Ptc1, 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 2 known negative factors in hedgehog signaling, showed only 36.6% (15 of 41) and 17.%1 (7 of 41) samples high expression. The hedgehog target genes, c-Myc and Bcl-2 were also detected and further confirmed activation of the signaling (Supplementary Fig. S1). For further validation of the results, we applied LCM to obtain tumor tissues from another 30 ICC samples to evaluate hedgehog components expression and found the similar expression pattern (Supplementary Fig. S2A). We detected some key genes’ copy number in fresh ICC tissues and found there few copy number changes in these genes (Supplementary Fig. S2B).

**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 (Supplementary Fig. S3). 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 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 conducted on the tumor samples to confirm positive staining cells were ICC cells (Supplementary Fig. S3G and S3H).
Association of Shh, Gli1, and Gli2 expression with the clinicopathologic 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 Union Internationale Contra Cancrum (UICC) $T$ 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 (Supplementary Table S1).

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-, 3-, and 5-year OS were 65.7%, 29.6%, and 14.8% for all of the patients in this study. On the basis of the each protein intratumoral densities, the patients were divided into 2 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 OS prognosis and a shorter DFS than those with low Gli1 expression (training set Fig. 2A and B; validation cohort Fig. 2E and F). The 1-, 3-, and 5- OS were 68.3%, 41.3%, and 20.6% for low
Gli1 expression patients in training cohort. While the high Gli1 expressers’ 1-, 3-, and 5- 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 and D; validation cohort Fig. 2G and H). The 1-, 3-, and 5- OS were 67.6%, 39.7%, and 22.1% for low Gli2 expression patients in training set. While the high Gli2 expressers’ 1-, 3-, and 5- 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 Supplementary Table S2. 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 histopathologic variables known to affect prognosis, a multivariate analysis was conducted to assess the independence of Gli1 and Gli2 prognosis. It was shown that Gli1, together with those of tumor size, intrahepatic metastasis, and 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 (Supplementary Fig. S4A and S4B). Furthermore, proliferation of Gli1-siRNA cells was evidently slowed down in RBE, HCCC9810, and QBC939 cells (Fig. 3A and Supplementary Fig. S4E). As shown in Fig. 3B, depleting Gli1 markedly reduced colony formation of RBE, HCCC9810, and QBC939. Stably transfected Gli1-overexpressed RBE

Figure 2. Kaplan–Meier curves for time to recurrence and OS 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 cutoff value of Gli1 or Gli2 level defined as the median of the cohort.
and found inhibition of Gli1 markedly induced cell apoptosis. Then, we detected the apoptosis in Lv-SiRNA-treated cells, whereas the control cell did not show effects. In cholangiocarcinoma cells, the AdSiGli1 significantly decreased the expression of Gli1 mRNA and protein (Supplementary Fig. S4C and S4D). 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 and E). We further tested the role of hedgehog...

<table>
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<tr>
<th>Variables</th>
<th>DFS</th>
<th>P value</th>
<th>OS</th>
<th>P value</th>
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<td><strong>Univariate analysis</strong></td>
<td></td>
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<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>
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<td>Gli 2 (high vs. low)</td>
<td>1.660 (1.085–2.540)</td>
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<td>1.978 (1.293–3.028)</td>
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<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>
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<td>Age (≥60 vs. ≤60 y)</td>
<td>1.280 (0.819–2.000)</td>
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<td>1.211 (0.777–1.886)</td>
<td>0.398</td>
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<td>Gender (male vs. female)</td>
<td>0.624 (0.391–0.998)</td>
<td>0.048</td>
<td>0.620 (0.368–0.991)</td>
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<td>Hepatitis B virus (negative vs. positive)</td>
<td>1.399 (0.916–2.315)</td>
<td>0.120</td>
<td>1.254 (0.822–1.913)</td>
<td>0.293</td>
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<td>Serum CA19-9 (&gt;37 vs. ≤37 U/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>
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<td>Serum CEA (&gt;10 vs. ≤10 ng/mL)</td>
<td>2.276 (1.165–4.448)</td>
<td>0.016</td>
<td>4.259 (2.125–8.533)</td>
<td>0.000</td>
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<td>Serum AFP (&gt;20 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>
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<td>Largest tumor size (&gt;5 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>
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<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>
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<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>
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<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>
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<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>
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<tr>
<td>Histologic 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>
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<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>
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<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>
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<tr>
<td>Resection status (R0 vs. R1/R2)</td>
<td>1.399 (0.775–2.524)</td>
<td>0.265</td>
<td>1.596 (0.886–2.878)</td>
<td>0.120</td>
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**Multivariate analysis**

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<th>P value</th>
<th>OS</th>
<th>P value</th>
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<tr>
<td>Gli 1 (high vs. low)</td>
<td>NA</td>
<td>NA</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>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Gender (male vs. female)</td>
<td>NA</td>
<td>NA</td>
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<td>NA</td>
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<tr>
<td>Serum CEA (&gt;10 vs. ≤10 ng/mL)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Largest tumor size (&gt;5 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>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Perineural invasion (negative vs. positive)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>UICC pT (1+2 vs. 3+4)</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>UICC stage (I+II vs. III+IV)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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</tbody>
</table>

**NOTE:** 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.

Abbreviation: CI, confidence interval.

*For Gli1, Gli2, and Shh median values were used as the cutoff point for definition of subgroups (low expression and high expression groups).
pathway activity in vivo by treating established subcutaneous xenograft tumors in nude mice with daily subcutaneous injections of cyclopamine or dimethyl sulfoxide (DMSO). After treatment with cyclopamine, the tumor growth was inhibited obviously (Supplementary Fig. S4G).

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 hedgehog components in some tumor types was reported (22–26). In the present study, we clearly show that many genes of hedgehog signaling upregulated in clinical ICC samples. The expression patterns of hedgehog 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 OS.

To the best of our knowledge, there was no report on the correlation of the hedgehog 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 and colleagues found that patients with esophageal squamous cell carcinoma 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 hedgehog pathway, Gli1 expression serves as a reliable indicator of activated hedgehog signaling, and

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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 extrahepatic cholangiocarcinoma cells by downregulating 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 hedgehog signaling in development was typically mediated through paracrine effects. Yauch and colleagues reported that hedgehog–Gli activation was required in the tissue mesenchyme surrounding pancreatic cancer cells to support tumor growth by paracrine effects (35). Fingas and colleagues reported an interesting work, which revealed that myofibroblast-derived platelet-derived growth factor (PDGF) promoting survival of cholangiocarcinoma cells (36). Therefore, the role of hedgehog paracrine signaling may be required for the progression of ICC, which needs to be further investigated.

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

**Disclosure of Potential Conflicts of Interest**
No potential conflicts of interest were disclosed.

**References**


**Authors’ Contributions**

Conception and design: Y.-X. Tan, L.-W. Dong

Development of methodology: Y.-X. Tan, Q. Wang

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y.-X. Tan, B.-G. Jiang, M. Wang

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Tang, Y.-X. Tan, S.-X. Li, G.-Z. Yang, Q. Wang, Z. Zhang, W.-P. Zhou

Writing, review, and/or revision of the manuscript: Y.-X. Tan, L.-W. Dong

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y.-X. Tan, Y.-F. Pan, C.-Z. Yang

Study supervision: Y.-X. Tan, H.-Y. Wang

**Grant Support**

Research was supported by grants from National Natural Science Foundation of China (81010075 and 81071681), Innovation Program of Shangh hai Municipal Education Commission (10ZZ53, 09ZZ82, and 91029732), the Funds for Creative Research Groups of China (81221061), and the State Key Project for Liver Cancer (2012ZX10002-009 and 2012ZX10002-010). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received February 4, 2012; revised January 24, 2013; accepted February 19, 2013; published online First April 14, 2013.


Clinical Cancer Research

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doi:10.1158/1078-0432.CCR-12-0349

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