Activation of the hedgehog pathway in human hepatocellular carcinomas

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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.

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 manufacturer’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 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 Cq values were analyzed in Microsoft Excel using the comparative C(T) method as described by the manufacturer (Applied Biosystems, Foster City, CA). The amount of target (2-DD CT) was obtained by normalization to an endogenous reference (18S RNA) and relative times, and the percentage from each counting was calculated.

**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 Chihiro 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) or Shh antibodies (1 μM) or Shh antibodies (1 μg/ml) was 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 indicated 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.
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

### Table I. Detection of Shh, PTC1 and Gli1 expression in HCC and in adjacent liver tissue by *in situ* hybridization

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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, PTC1 expression in primary HCCs. *In situ* hybridization detection of Gli1 (A) and PTC1 (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 PTC1 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 PTC1 (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
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
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,
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.

**References**


