The sonic hedgehog pathway as a treatment target for extrahepatic biliary tract cancer

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Abstract. Sonic hedgehog (SHh) signaling is essential for normal development of the human gastrointestinal (GI) tract and is reported to be aberrantly activated in GI cancers. However, the association between SHh signaling and extrahepatic biliary tract cancer is not clearly understood. In this study, we evaluated the activities of SHh family proteins and their downstream signals in extrahepatic biliary tract cancer. The activity of the SHh pathway was analyzed in established human extrahepatic biliary tract cell lines and human cancer tissues using RT-PCR and immunohistochemistry. We also evaluated the effects of suppressing the SHh pathway with cyclopamine and siRNA. The SHh, Smo and Gli-1 genes were overexpressed in extrahepatic biliary tract cancer cell lines and six extrahepatic biliary tract cancer tissues compared to the levels in normal biliary tract tissues. The degrees of SHh and Gli-1 expression were independent of tumor stage and cancer cell differentiation. SHh pathway suppression with cyclopamine or siRNA inhibited proliferation of extrahepatic biliary tract cancer cell lines. In conclusion, the SHh pathway is highly activated in extrahepatic biliary tract cancer and is a potential anticancer drug target.

Introduction

Biliary tract cancers are malignancies of the epithelial lining of the intrahepatic and extrahepatic biliary tracts. They are relatively rare in Western countries, but are common in Korea. They are the sixth most frequent cause of cancer-related morality in Korea, with a national mortality rate that is almost equal to that of pancreatic cancer. Surgery is the only curative treatment method; however, surgery is possible for less than 30% of patients at the time of diagnosis and, even with curative resection, the majority of patients experience relapse within 2 years (1). For patients with unresectable disease, the situation is even worse, since a chemotherapeutic regimen provides only minimal clinical benefit. With these grave conditions, the overall survival for biliary tract cancer is approximately 6 months (2). Additional clinical trials and development of novel drugs are urgently required to improve outcomes, but have been hindered by a lack of interest.

Developing better anticancer treatments requires understanding of the molecular pathways in cancer cells to identify potential targets. Research on other types of cancer has shown that cancer stemness-associated pathways may be potential drug targets for biliary tract cancers. Stem cell pathways include the Sonic hedgehog (SHh), Notch, Wnt, TGFβ and other pathways that are normally activated during the embryonic development period and in adult tissue regeneration, but that are also aberrantly activated in numerous types of cancer. In normal tissue, these pathways act in self-renewal and differentiation, while their activation in tumor cells is involved in cancer maintenance, development, carcinogenesis, drug resistance, recurrence and metastasis. Stem cell pathways may have similar roles in biliary tract cancers and may be potential treatment targets.

Among these stem cell pathways, the SHh signaling pathway is crucial in pre-natal development and in adults for controlling differentiation, maintaining stem cell niches and determining the cellular response to injury. The SHh pathway is particularly critical to gastrointestinal development and function. Binding of the SHh protein to its receptor results in depression of the Smoothened (Smo) gene. Activated Smo is thought to initiate a series of signal cascades, including activation of the glioma-associated (Gli) family of transcription factors. The activation of the SHh pathway has been observed in a number of types of cancer (3-5); in addition, the SHh signaling pathway and the development and proliferation of biliary tract cancer are reported to be connected (6,7).

Biliary tract cancer is composed of tumors with different etiologies, behaviors, treatment responses and prognoses (8).

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Thus, to truly determine the biologic significance of the SHh pathway activity in biliary tract cancers, it should be analyzed according to tumor location. In this study, we investigated the possibility of the SHh pathway as a therapeutic target in extrahepatic biliary tract cancer by evaluating the activity of the pathway in cancer cell lines and human cancer tissues and by suppressing the pathway to determine its role in extrahepatic biliary tract cancer.

Materials and methods

Clinical samples. Cancer and adjacent normal tissues from surgical specimens of 6 patients with extrahepatic biliary tract cancer were used for PCR and Western blotting, and 42 tissue and adjacent normal tissue blocks from patients who underwent curative resection were used for immunohistochemical staining.

Cell cultures and reagents. The human extrahepatic biliary tract adenocarcinoma cell lines used in this study were SNU245 (origin: common bile duct), SNU478 (origin: ampulla of vater), SNU869 (origin: ampulla of vater) and SNU1196 (origin: hepatic bifurcation) from the Korean Cell Line Bank (Seoul, Korea). Cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum and were maintained in humidified incubators at 37˚C in an atmosphere of 5% CO_2 and 95% air.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from tissues and cultured cell lines using an RNeasy mini kit (Qiagen Inc., Valencia, CA, USA). Primers were 5'-GAA TCG CTA CCC TGC TGT TA-3' and 5'-TGA GCA GGT GGA AGT AGG AG-3' for Smo, 5'-CCT ACC AGA GTA CCA AGT TT-3' and 5'-AGA GTC CAG GGG GTT ACA TA-3' for Gli-1, 5'-AGA AGA AGG AGT TTG TGT GA-3' and 5'-CAG GTG TGT CTT CAG GTT CT-3' for Gli-2, and 5'-GGC ATC CTC ACC CTG AAG TA-3' and 5'-GGG GTG TTG AAG GTC TCA AA-3' for β-actin. Amplification occurred over 28 cycles at a 58˚C (β-actin and Smo) or 56˚C (Gli-1 and Gli-2) annealing temperature with a 72˚C extension. RT-PCR products were separated on ethidium bromide-stained 1.5% agarose gels. RT-PCR product sizes were 314 bp for Smo, 347 bp for Gli-1, 176 bp for Gli-2 and 203 bp for β-actin.

Western blotting. Cells were washed once in cold phosphate-buffered saline (PBS) and lysed in a buffer containing 70 mM β-glycerophosphate (pH 7.2), 0.6 mM Na-vanadate, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 0.5% Triton X100, 0.5% NP-40, 0.2 mM PMSF and protease inhibitors. Samples were separated using SDS-PAGE and transferred to a PVDF membrane (Millipore Corporation, Billerica, MA, USA). Membranes were blocked for 1 h with 5% nonfat milk in Tris-buffered saline/0.05% Tween-20 at room temperature, and probed overnight with primary antibodies against SHh, Pached (Ptch), Smo, Gli-1, Gli-2 and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) for 1 h and washed a number of times. Complexes were visualized using an enhanced chemiluminescence system (Pierce, Rockford, IL, USA).

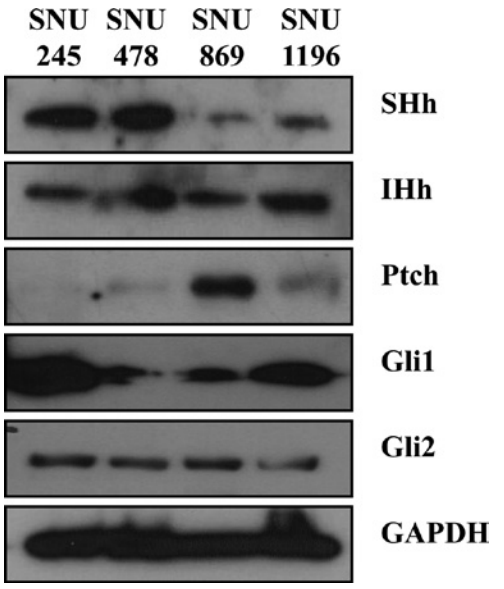

Figure 1. Western blot analysis of extrahepatic biliary tract cancer cell lines: SHh, IHh and Gli-1 were overexpressed in all four extrahepatic cholangiocarcinoma cell lines, while Ptch was overexpressed only in the SNU 869 cell line.

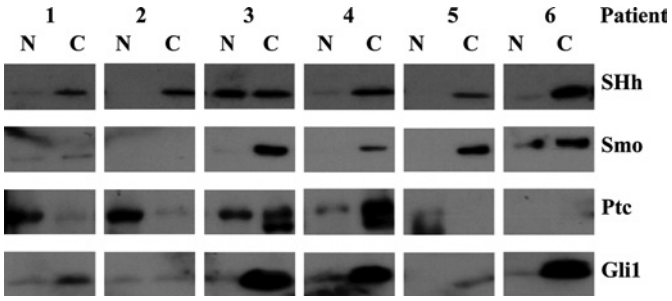

Figure 2. Western blot analysis of extrahepatic biliary tract cancer tissues to detect expression of SHh signaling protein. N, normal tissue; C, cancer tissue. Compared to adjacent normal tissue, SHh, Smo and Gli-1 were overexpressed in the cancer tissue, while Ptch showed reduced expression.

Immunohistochemistry. The immunoactivity of the SHh signaling pathway was evaluated in 42 extrahepatic biliary tract cancer tissues and adjacent normal tissues that were heated for 1 h. Tissues were dewaxed with xylene, rehydrated with alcohol and heated in pressure jars filled with 10 mM citrate buffer using a microwave for 10 min. Following heating, slides were cooled in water for 15 min and washed in PBS before incubating overnight with specific primary rabbit polyclonal antibodies against SHh, Gli-1 or Ptch (Santa Cruz Biotechnology). Each antibody was diluted 1:100. Following incubation with primary antibody overnight at 4˚C, samples were treated with an Envision kit (DakoCytomation, Carpinteria, CA, USA), incubated with 3,3'-diaminobenzidine (DakoCytomation) and counterstained with hematoxylin (Sigma, St. Louis, MO, USA). Results were subdivided into three groups according to the percentage of positive staining by tumor volume (grade 0, <5%; grade 1, 5-50%; grade 2, >50%) and analyzed.

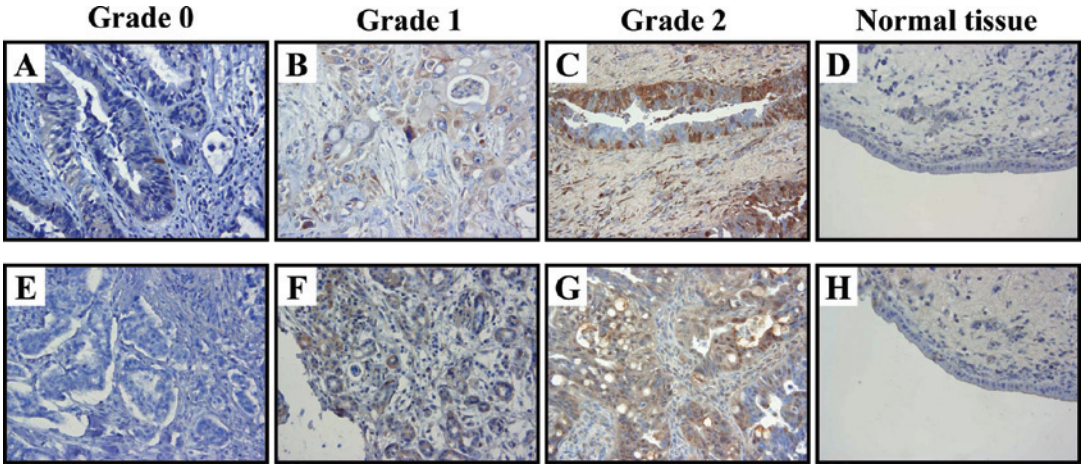

Figure 3. Examples of immunohistochemical staining grades in extrahepatic biliary tract cancer and normal tissues. (A-D) SHh; (E-H) Gli-1.

Proliferation assay. Cyclopamine-KAAD (Calbiochem, CA, USA) was dissolved in 100% methanol. Extrahepatic biliary tract cancer cells were treated with 5 or 10 nM of cyclopamine-KAAD, Smo siRNA or control siRNA (Santa Cruz Biotechnology) according to the manufacturer's instructions. Gene and protein expression of Smo, Gli-1 and Gli-2 in the SNU478 and SNU1196 cell lines was analyzed using RT-PCR and Western blotting. Cells ($1x10^3$/well) were seeded into 96-well plates and grown for 24 h. Cultures were grouped into seven (0-6) treatment groups (n=3 wells/group): group 0, control; group 1, cyclopamine-KAAD 1 nM/ml; group 2, cyclopamine-KAAD 5 nM/l; group 3, cyclopamine-KAAD 10 nM/l; group 4, cyclopamine-KAAD 25 nM/l; group 5, cyclopamine-KAAD 50 nM/l; and group 6, cyclopamine-KAAD 100 nM/l. Following incubation for 72 h, a colorimetric reaction was performed using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] (Chemicon, Temecula, CA, USA) at a concentration of 5 mg/ml, and then the cells were incubated for 3 h. Absorbance was measured at 570 nm using an ELISA plate reader to estimate the effects of each reagent on cell proliferation. The drug 50% inhibitory concentration (IC_{50}) that inhibited cell proliferation by 50% was estimated from individual inhibition curves.

Results

Activated SHh pathway in extrahepatic biliary tract cancers. Western blot analysis showed that all four extrahepatic biliary tract cell lines expressed SHh, Gli-1 and Gli-2. However, the expression of Ptch was observed only in the SNU869 cell line (Fig. 1). A Western blot analysis of tissue samples also showed that the expression of SHh was higher in all cancer tissues than it was in adjacent normal tissues. The expression of Smo was higher in four of six tumor tissues than in adjacent normal tissue. Gli-1 was also overexpressed in five of six tumor tissues compared to the levels in normal tissues (Fig. 2). However, the expression of Ptch was increased only in two of six tumor tissues.

Cytoplasmic staining of SHh and nuclear staining of Gli-1 via immunohistochemistry were used as a marker of SHh pathway activation. Fig. 3 shows examples from each group. SHh and Gli-1 showed higher expression in cancer cells than in adjacent normal bile duct epithelial cells. In 36 (85.7%) of 42 cancer tissues, the levels of SHh expression were ≥ grade 1. However, SHh was not expressed in 27 (64.3%) of 42 adjacent normal bile duct tissues. The expression of Gli-1 was similar to that of SHh (Table I). The levels of SHh and Gli-1 expression were independent of tumor stage and cancer cell differentiation (Table II).

Table I. Expression of Sonic hedgehog (SHh) signaling molecules in extrahepatic biliary tract cancer tissue using immunohistochemical staining [no. of patients (%)].

Antibody	Grade of staining		
	0	1	2
SHh			
Normal tissue	27 (64.3)	11 (26.2)	4 (9.5)
Cancer tissue	6 (14.3)	20 (47.6)	16 (38.1)
Gli-1			
Normal tissue	22 (53.7)	16 (39.0)	3 (7.3)
Cancer tissue	12 (29.3)	22 (53.7)	7 (17.1)

Suppression of the SHh pathway with cyclopamine and siRNA. To evaluate the potential of the SHh pathway as a therapeutic target in extrahepatic biliary tract cancer, we suppressed the pathway with cyclopamine or siRNA against Smo in the SNU478 and SNU1196 cell lines. Western blotting confirmed decreased expression of Smo and Gli-1 with Smo siRNA (Fig. 4). Western blotting and RT-PCR revealed that cyclopamine also dose-dependently decreased Smo, Gli-1 and Gli-2.

The toxicity of cyclopamine was evaluated using an MTT assay, which demonstrated that 0.5-50 μM of cyclopamine-KAAD had a concentration-dependent cytotoxic effect on the SNU478 and SNT1196 cells (Fig. 5). Cyclopamine-KAAD had a cytotoxic effect on SNU478 cells and caused a 21.6% cell growth inhibition at 1 μM and 67.9% at a concentration of 2.5 μM. In SNU1196 cells, cyclopamine-KAAD caused a 45.4% cell growth inhibition at 2.5 μM and 63.1% at 5 μM.

Table II. Correlation between immunohistochemical staining grade of extrahepatic biliary tract cancer and patient characteristics.

Characteristic	SHh staining grade (n=42; %)			Gli-1 staining grade (n=41; %)		
	0	1	2	0	1	2
Gender						
Male	2 (9)	11 (50)	9 (41)	9 (41)	9 (41)	4 (18)
Female	4 (20)	8 (40)	8 (40)	3 (16)	12 (63)	4 (21)
Age (years)						
<63	3 (16)	9 (48)	7 (36)	5 (29)	7 (42)	5 (29)
≥63	3 (14)	10 (43)	10 (43)	7 (29)	14 (58)	3 (13)
Stage						
1	1 (10)	5 (50)	4 (40)	2 (20)	7 (70)	1 (10)
2	5 (16)	14 (44)	13 (40)	10 (32)	14 (45)	7 (23)
Differentiation						
Good	4 (24)	6 (38)	6 (38)	4 (27)	8 (53)	3 (20)
Moderate to poor	2 (8)	13 (50)	11 (42)	8 (31)	13 (50)	5 (19)

Immunohistochemistry was scored as: grade 0, <5%; grade 1, 5-50%; grade 2, >50%. P>0.05, for each SHh and Gli-1.

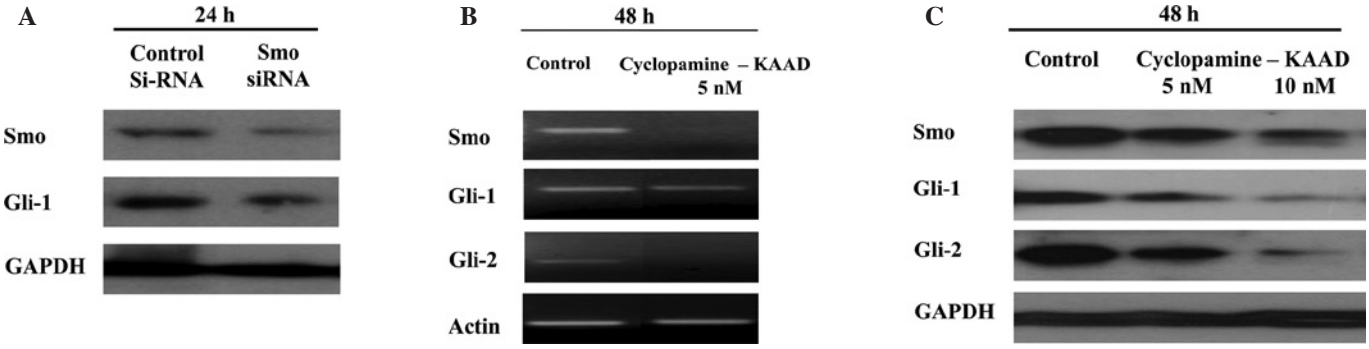

Figure 4. RT-PCR and Western blotting of SNU478 cells following treatment with siRNA against Smo or cyclopamine-KAAD. (A) In Western blot analysis, Smo and Gli-1 expression was inhibited by Smo siRNA. (B) In RT-PCR, Smo, Gli-1 and Gli-2 expression was inhibited by cyclopamine-KAAD. (C) In Western blots, Smo, Gli-1 and Gli-2 expression was inhibited by cyclopamine-KAAD in a dose-dependent manner.

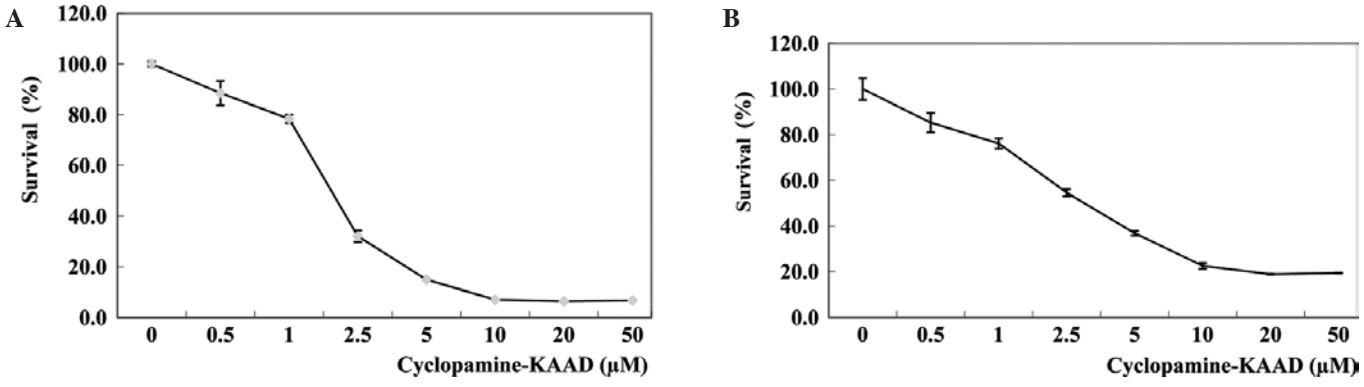

Figure 5. MTT assay results for extrahepatic biliary tract cancer cell lines. Cancer cell proliferation was suppressed by the Smo inhibitor cyclopamine-KAAD. (A) SNU478 cell line; (B) SNU 1196 cell line.

Discussion

The SHh pathway has been suggested as a potential treatment target for a number of gastrointestinal tract cancers. Cancer cell lines derived from the esophagus, stomach, pancreas and biliary tract expressed SHh, IHh, Ptch and Gli-1, and cyclopamine treatment reduced the growth of tumor cell lines (6). Since biliary tract tumors have heterogeneous characteristics, consistent activation of the SHh pathway was required to be demonstrated in extrahepatic biliary tract cancer samples. If

currently tested drugs targeting the SHh pathway are effective against other gastrointestinal cancers, they could also be used against extrahepatic bile duct cancer. We showed that the SHh pathway was highly activated not only in established cancer cell lines, but also in surgically resected tumor tissues. The SHh pathway was also shown to be involved in the growth of extrahepatic biliary tract cancer.

Our results are consistent with those of previous studies. Usually, activation of the SHh pathway is associated with increased expression of Ptch (6); however, unlike other gastrointestinal cancers, Ptch expression was detected only rarely in the extrahepatic biliary tract cancer cell lines and tumor tissues. In ligand-associated SHh pathway activation, Ptch has an inhibitory effect on Smo in gastrointestinal cancers (6,9,10). However in certain types of cancer, such as medulloblastomas and ovarian cancers, the SHh pathway is activated independent of its ligand (11,12). Usually, these types of cancer have constitutive activation of a receptor or downstream signaling molecule. The ligand-independent pathway activities are caused by mutations that result in Smo insensitivity to Ptch or mutations that inactivate Ptch. Ptch transcription is induced by the SHh pathway activity, thus generating a negative feedback. Similar to medulloblastomas and ovarian cancers, the observed down-regulation of Ptch in extrahepatic biliary tract cancer cell lines and tissues suggested that extrahepatic biliary tract cancer showed ligand-independent activation of Smo and SHh signals.

SHh and Gli-1 were more highly expressed in extrahepatic biliary tract cancer tissues than they were in adjacent normal tissues. In certain reports, SHh pathway activation is associated with the tumor stage or cell differentiation (13,14); however, our results with extrahepatic biliary tract cancers showed that SHh pathway activation was independent of tumor stage and cell differentiation. SHh pathway activation in extrahepatic biliary tract cancer may be associated with inflammation since these types of cancer usually have concomitant cholangitis, and the SHh pathway is usually increased in chronic inflammation (15). Further studies are required to evaluate the effect of inflammation on the SHh pathway in biliary tract cancer.

To investigate the functional activities of the SHh pathway, we inhibited the pathway at the level of Smo using siRNA and cyclopamine (16), a Smo antagonist (17,18). siRNA and cyclopamine suppressed the SHh pathway, and the cancer cell lines were suppressed by cyclopamine. Evidence suggests that the SHh pathway may be a therapeutic target for gastrointestinal cancers (4,6,19). However, to date it was not clear whether SHh signaling could be a therapeutic target for biliary tract cancer. Our results indicate that inhibiting the SHh pathway may be an effective therapeutic strategy for patients with extrahepatic biliary tract cancer. However, SHh signaling is complex, and further studies are required to confirm our results (20).

The SHh pathway was associated with cell proliferation and could be regarded as a potential anticancer drug target in extrahepatic biliary tract cancer. Further studies will help to understand the mechanism behind inhibition of the SHh pathway and cancer cell death.

Acknowledgements

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