Blockade of the sonic hedgehog pathway effectively inhibits the growth of hepatoma in mice: An *in vivo* study

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Abstract. Hepatocellular carcinoma (HCC), a worldwide malignancy, is prevalent in Asian countries. For individuals with unresectable HCC, the effect of chemotherapy or the present target therapy is limited. There is an urgent need to find innovative new therapies. It is believed that sonic hedgehog (*Shh*) pathway activation may be essential for hepatocarcinogenesis. In the present study, we conducted an *in vivo* animal study using an *Shh* pathway inhibitor to elucidate the effect of treatment upon mice with HCC. Eighty C57BL/6 mice were divided into 4 groups (groups A, B, C and D, with group A serving as a control; n=20 for each). We injected mouse hepatoma Mistheton Lectin-1 cells ($5x10^6$ cells/20 μl) into the left liver of each mouse in groups B, C and D. In the second week, we analyzed each mouse to assess the tumor growth status. Following the tumor injection, group B did not receive any additional intraperitoneal (i.p.) injection, group C received cyclopamine 10 mg/kg/day i.p. and group D received cyclopamine 30 mg/kg/day i.p. every day for 10 days. After an interval of 4 weeks, harvesting and analysis of the liver was performed for each mouse. Tumor size measurement and real-time PCR of *Shh* pathway factors (*Shh*, *Ptch-1, Gli-1* and *Smoh*) for livers of group A and tumors of group B, C and D were undertaken. The decrease in the tumor size of group D was found to be statistically significant (P=0.047) when compared with groups B or C. The decrease of *Shh* mRNA of both groups C and D had borderline significance when compared with group B. However, *Gli-1* mRNA of group D has statistically significant difference (P=0.044) when compared with group A, B or C. Inhibition of the *Shh* pathway significantly decreases the size and *Gli-1* mRNA expression of the tumor. The *Shh* pathway may be an effective treatment target for HCC in the future.

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Introduction

Hepatocellular carcinoma (HCC), one of the most prevalent malignancies worldwide, is recently rapidly increasing in the United States, and is particularly prevalent in Taiwan and other Asian counties (1-5). For individuals with unresectable HCC, the effect of either chemotherapy or the present target therapy is limited. The need to find new therapies is urgent.

Previous studies have suggested that abnormal activation of the sonic hedgehog (*Shh*) signaling pathway may be essential for carcinogenesis in certain cancer types, including HCC (6-18). Cyclopamine, a well-known antagonist of Smoothened (*Smoh*), may inhibit the *Shh* pathway. The effect of cyclopamine on hepatocarcinogenesis has been described in a previous study (19). However, the *in vivo* effect remains unknown. We conducted this study to investigate the treatment effect of cyclopamine upon HCC in an *in vivo* model of mice.

Materials and methods

Treatment groups. Eighty C57BL/6 mice (6-8 weeks old, 19-24 g) were purchased and divided into 4 groups (A, B, C and D) with 20 mice in each. Group A formed the control group. Under isoflurane general anesthesia, we injected mouse hepatoma cells, i.e., Mistheton Lectin-1 (ML-1) cells ($5x10^6$ cells/20 μl), into the left liver of mice in groups B, C and D. In the second week, we analyzed each mouse to assess the tumor growth status. Following the initial tumor injection, group B did not receive any additional drug injections. Group C received cyclopamine 10 mg/kg/day i.p and group D received cyclopamine 30 mg/kg/day i.p. The injections were administered every day for 10 days. After an interval of 4 weeks, exploration and harvesting of the liver was performed for each group. The tumor size was measured for groups B, C and D. Real-time PCR analysis of *Shh* pathway factors [*Shh*, patched homolog-1 (*Ptch-1*), glioma-associated oncogene homolog-1 (*Gli-1*) and *Smoh*) of the livers in group A and of the tumors in groups B, C and D were undertaken. The experiment was conducted under the Guidelines for the Care and Use of Laboratory Animals of the Far Eastern Memorial Hospital, Taiwan. The institutional licensing committee had approved the experiments undertaken.

Definition of effective reduction of tumor size following treatment. The maximal diameter of the tumor size of each mouse (groups B, C and D) was measured at the final evaluation. Effective reduction of the tumor size following treatment was defined if there was a statistically significant reduction in the tumor size as measured at the end of the study compared to the tumor size prior to treatment (measured in the second week after ML-1 cell injection).

Detection of mouse mRNA of Shh, Ptch-1, Gli-1 and Smoh. The examination included extraction of RNA and reverse transcription, and amplification of cDNA of *Shh*, *Ptch-1*, *Gli-1*, *Smoh* and housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) by real-time PCR.

Extraction of RNA and reverse transcription PCR. We homogenized each resected cancer and liver tissue completely in 1 ml TRIzol® reagent (Invitrogen, Carlsbad, CA, USA), and added 0.2 ml chloroform and agitated it vigorously by hand for 15-30 sec, then incubated them on ice for 20 min. The samples were then centrifuged at 13,000 rpm for 20 min at 4˚C. Following centrifugation, the mixture was separated into a lower red, phenol-chloroform phase, an interphase and a colorless upper aqueous phase. RNA remained exclusively in the aqueous phase. We transferred the aqueous phase to a fresh tube, and precipitated the RNA from the aqueous phase by mixing in 0.5 ml of isopropanol. The samples were incubated on ice for 20 min and were centrifuged at 13,000 rpm for 20 min at 4˚C. The RNA precipitation, often invisible before centrifugation, formed a gel-like pellet on the side and bottom of the tube. We removed the supernatant and washed the RNA pellet once with 75% ethanol, adding at least 1 ml 75% ethanol. We centrifuged it at 13,000 rpm for 5 min at 4˚C. The RNA pellet was dried and RNA was dissolved in RNase-free water. Then it was incubated for 10 min at 60˚C and was stored at -80˚C.

cDNA was synthesized from 2 mg mRNA. The reverse transcription reaction solution consisted of 2.0 ml 10X RT buffer, 0.8 ml 100 mM dNTP mixed with 2.0 ml 10X RT random primers and 1.0 ml MultiScribe™ Reverse Transcriptase (Applied Biosystems, Foster City, CA, USA). The RNA solution was mixed with the reverse transcription reaction solution (total volume 20 ml) and incubated at 25˚C for 10 min, 37˚C for 120 min and 85˚C for 5 sec. It was stored at -20˚C.

Amplification of cDNA of Shh, Ptch-1, Gli-1, Smoh and GAPDH by real-time PCR. Total RNA was extracted from each liver tissue and HCC tissue using TRIzol reagent. RT-PCR was performed using high-capacity cDNA reverse transcription kits (Applied Biosystems). In brief, 2-5 μg total RNA was used in a 20-μl reverse transcription assay. Subsequently, the cDNA was diluted at 1:4 for real-time PCR assays which were carried out in a 96-well plate in the LightCycler 480 (Roche Diagnostics, Mannheim, Germany) using SYBR-Green I Master dye (Roche Diagnostics). Each real-time PCR assay (10 μl) contained 3 μl water, 0.5 μl forward and reverse primers, respectively, 5 μl 2X SYBR-Green I Master and 1 μl diluted cDNA. All primer sequences used for real-time analysis are listed in Table I. Real-time PCR parameters were cycled as follows: hot start at 95˚C for 1 min, followed by 45 cycles of denaturing at 95˚C for 10 sec, annealing at 58˚C for 5 sec and extension at 72˚C for 20 sec. PCR products were detected using 2% agarose gel to confirm the expected sizes. To normalize the total amount of cDNA in each reaction, *GAPDH* was coamplified as the internal control. Each sample was analyzed 3 times and quantified with the analysis software for LightCycler (Roche Diagnostics).

Table I. Sequences of primer pairs.

Gene	Direction	Primers (5'-3')
GAPDH	sense	5'-CACCACCAACTGCTTAG-3'
	antisense	5'-CTTCACCACCTTCTTGATG-3'
Shh	sense	5'-AAAGCTGACCCCTTTAGCCTA-3'
	antisense	5'-TTCGGAGTTTCTTGTGATCTTCC-3'
Ptch-1	sense	5'-CCGTTCAGCTCCGCACAGA-3'
	antisense	5'-CTCACTCGGGTGGTCCCATAAA-3'
Gli-1	sense	5'-TGTGGCGAATAGACAGAGGT-3'
	antisense	5'-TGCCAGATATGCTTCAGCA-3'
Smoh	sense	5'-GAGCGTAGCTTCCGGGACTA-3'
	antisense	5'-CTGGGCCGATTCTTGATCTCA-3'

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; *Shh*, sonic hedgehog; *Ptch-1*, patched homolog-1; *Gli-1*, glioma-associated oncogene homolog-1; *Smoh*, smoothened homolog.

Statistical analysis. Comparisons between groups were performed with a Chi-square test (or Fisher's exact test) for continuous variables. The least significant difference (LSD) pair-wise multiple comparison was used for multivariate analysis of associated factors. All statistical analyses were performed using the SPSS version 17.0 (Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant result.

Results

No tumors were observed in the livers in group A mice at any point in this study. At the end of the second week following injection of ML-1 cells, tumors developed successfully in left lobe of the liver of all the mice in groups B, C and D. At the end of the study, the reduction of the tumor size in group D was found to be significant, from 0.152±0.219 cm^2 (mean ± SD) before treatment to 0.003±0.009 cm^2 (mean ± SD) after treatment (P=0.047). The tumor size in group C reduced from 0.152±0.219 cm^2 to 0.071±0.187 cm^2 without statistical significance (P=0.267). Fig. 1 shows the decrease of tumor size in one mouse from group D.

Table II shows the mRNA expression of *Shh, Ptch-1, Gli-1* and *Smoh* in the 4 groups. Compared with group B, the value of *Shh* mRNA of HCC in groups C and D decreased. However, the difference of each had only borderline significance (P=0.062 and 0.071; Table II). Compared with group B, the decrease of *Ptch-1* mRNA expression in groups C and D

Table II. Comparison of the mRNA expression of *Shh*, *Ptch-1*, *Gli-1* and *Smoh* of liver (group A) and HCC (groups B, C and D).

Group	*Shh*	*Ptch-1*	*Gli-1*	*Smoh*
Group A				
Median	0.58	1.715	0.74	2.87
Mean ± SD	0.5575±0.0810	1.7325±0.4918	0.7125±0.3165	3.1333±1.2952
95% CI	0.4286-0.6864	0.9500-2.5150	0.2089-1.2161	-0.0842-6.3509
Range	0.45-0.62	1.22-2.28	0.31-1.06	1.99-4.54
Group B				
Median	1.23	1.29	1.22	2.905
Mean ± SD	1.2720±0.4535	1.9920±1.8249	1.2825±0.1839	3.0875±1.5321
95% CI	0.7090-1.8350	-0.2739-4.2579	0.9898-1.5752	0.6496-5.5254
Range	0.67-1.89	0.85-5.22	1.15-1.54	1.59-4.95
Group C				
Median	0.595	0.685	1.07	2.145
Mean ± SD	0.7175±0.4310	0.7950±0.3397	1.1600±0.2762	3.2125±3.2488
95% CI	0.0317-1.4033	0.2545-1.3355	0.4738-1.8462	-1.9571-8.3821
Range	0.36-1.32	0.52-1.29	0.94-1.47	0.75-7.81
Group D				
Median	0.77	0.61	0.94	1.4475
Mean ± SD	0.7700±0.2993	0.6840±0.3818	0.9380±0.2128	0.8640±0.6661
95% CI	0.3984-1.1416	0.2099-1.1581	0.6738-1.2022	0.0369-1.6911
Range	0.43-1.12	0.23-1.10	0.71-1.15	0.30-1.82
P-value	B vs C: 0.062 B vs D: 0.071 C vs D: 0.848	B vs C: 0.145 B vs D: 0.097 C vs D: 0.887	B vs C: 0.484 B vs D: 0.044 C vs D: 0.200	B vs C: 0.932 B vs D: 0.130 C vs D: 0.112

Group A, control group without ML-1 cell implantation or drug treatment. Group B, ML-1 cell implantation, without any treatment. Group C, ML-1 cell implantation and treatment with cyclopamine 10 mg/kg/per day for 7 days. Group D, ML-1 cell implantation and treatment with cyclopamine 30 mg/kg/per day for 7 days. HCC, hepatocellular carcinoma; *Shh*, sonic hedgehog; *Ptch-1*, patched homolog-1; *Gli-1*, glioma-associated oncogene homolog-1; *Smoh*, smoothened homolog; SD, standard deviation; 95% CI, 95% confidence interval.

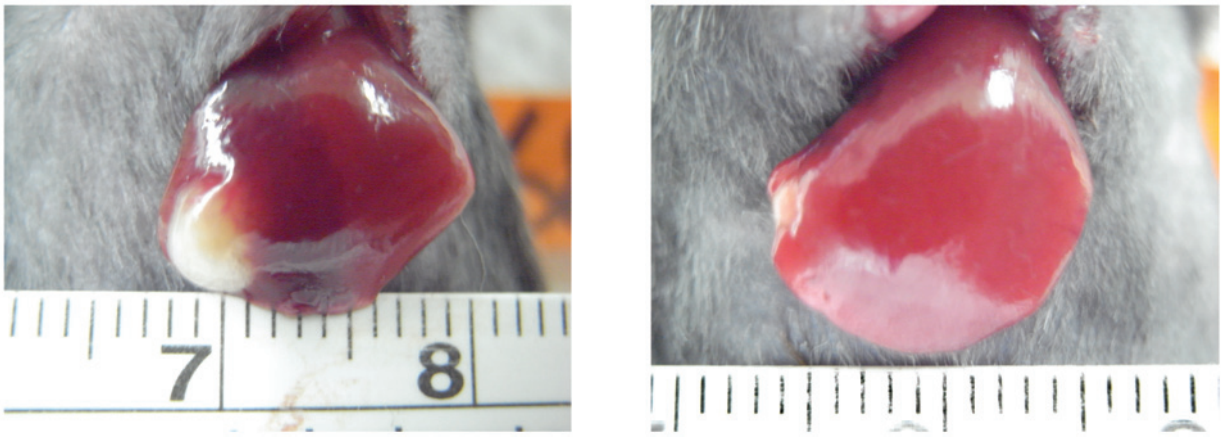

Figure 1. The size of liver tumor of one mouse in group D decreased from 3x2 mm^2 (left) to 1.2x1 mm^2 (right). Left: at second week after ML-1 cell injection, before cyclopamine treatment. Right: at fourth week after treatment with cyclopamine (i.p. 30 mg/kg/day x 10 days)

also had no statistical significance. However, compared with group B, the decrease of *Gli-1* mRNA in group D had statistical significance (P=0.044). Compared with group B, the decrease of *Smoh* mRNA in groups C and D was not statistically significant (Table II).

Discussion

In this study, we found that cyclopamine treatment, either at a low or high dose, may decrease the size of liver tumors in mice *in vivo*. The effect of high-dose therapy was significant.

We successfully established the growth of ML-1 hepatoma in the livers of groups B, C and D mice. A higher expression level of *Shh*, *Gli-1* and *Smoh* mRNA was observed in group B when compared with group A, which suggests that the activation of the *Shh* pathway occurred during HCC development in mice. This corresponds with certain authors' findings that compared with paired adjacent noncancerous liver tissue, *Shh*, *Ptch-1*, *Gli-1* and *Smoh* were overexpressed in human HCC tissues (17,20). Similarly, Patil *et al* used quantitative real-time RT-PCR and revealed an increased level of expression of *Gli-1* and *Smoh* in HCC samples compared with non-tumor liver tissues (16). Che *et al* found that in over 50% of human HCC, the mRNA of *Shh* pathway target genes *Ptch-1*, *Gli-1* and *Smoh* were expressed (17). Tada *et al* demonstrated that hedgehog signaling components were expressed in hepatoma cell lines in various degrees (21). These findings suggested that the hedgehog pathway was frequently activated or deregulated in human HCCs (14-17,21). *In vitro*, certain authors considered that some hedgehog signal-responsive progenitor cells function as cancer stem cells, leading to carcinogenesis (22-24).

The detailed molecular mechanisms and the effect of the timing of *Shh* pathway activation upon HCC are not well understood. Some authors have hypothesized that activation of the *Shh* pathway is important both in the development and the progression of HCC (14-18). Cheng *et al* found that the *Shh* signaling pathway correlated with the proliferation and invasiveness of HCC cells (20). In addition, some authors reported an association between the factors of *Shh* signaling pathways and invasiveness of human HCC (17,20).

Tada *et al* regarded the overexpression of *Smoh* or *Shh* as being positive regulators and the major trigger for the activation of this signaling pathway (21). The authors demonstrated that overexpression and/or tumorigenic activation of the *Smoh* proto-oncogene mediates c-myc overexpression, which plays a critical role in hepatocarcinogenesis (21). *Smoh* has been suggested as being a prognostic factor in hepatocarcinogenesis (21).

Cyclopamine is the inhibitor of *Smoh*. Cyclopamine has been reported to inhibit the growth of HCC cells or hepatoblastoma cells (19,25,26). Chen *et al* revealed that cyclopamine markably decreased cell viability, induced apoptosis and downregulated Bcl-2 expression in HCC cells (19). Kim *et al* treated three hepatoma cell lines with KAAD-cyclopamine, resulting in a decrease of the expression of hedgehog target genes and cell growth, leading to apoptosis (25). Cheng *et al* showed that the blockade of the *Shh* signaling pathway by KAAD-cyclopamine induced a reduction of DNA synthesis leading to a marked inhibition of cell growth and a significant attenuation in invasiveness and motility of HCC cells (20). Collectively, the studies support the hypothesis that inhibition of the *Shh* pathway by cyclopamine may inhibit both the development and invasiveness of HCC.

However, the majority of these studies were carried out *in vitro*. By contrast, our present study is *in vivo*.

From our study, high-dose cyclopamine therapy not only effectively decreases the tumor size but also significantly decreases the expression of *Gli-1* mRNA in the tumors. The reason for the significant decrease of *Gli-1* mRNA and not the mRNA of *Smoh*, *Ptch-1* or *Smoh* is unknown. We attribute this result to three possible mechanisms.

The first is that the interactions among these factors of the *Shh* pathways are complex. *Ptch-1* activation predisposes a cell to proliferative and expansive behavior (22,27). Some elements of the interaction between *Smoh* and *Ptch-1* are not fully understood. *Smoh* is an intracellular substrate that migrates to the cellular membrane where it is activated following engagement of *Ptch-1* by *Shh*. At the cellular membrane, the activated *Smoh* triggers the downstream transcription of *Gli-1* proteins (22,27). Aberrant activation of the Shh pathway leads *Gli-1* into the nucleus to promote gene transcription and to maintain the biological behaviors of cancer cells.

However, the change of the mRNA expression may be dynamic. The timing of tumor harvesting affects the values of the factor.

The second mechanism may be that the significance of *Shh* pathway activation may be different among different stages of the same cancer and among different malignancies at the same stage. For example, a previous study reported that the proliferation of extrahepatic biliary tract cancer cell lines could also be suppressed by inhibition of the *Shh* pathway (28). However, the degrees of *Shh* and *Gli-1* expression were independent of tumor stage and cancer cell differentiation (28). Activation of the *Shh* pathway also occurs in different stages of the same cancer. Huang *et al* suggested that the activation occurs in the early stage of HCC (14), whereas Thayer *et al* considered the hedgehog is both an early and late mediator in pancreatic carcinogenesis (13). The activation of the *Shh* pathway occurring in advanced stages of other cancers is also noted (8,10,13). The detailed cause of these discrepancies needs further elucidation.

The third possibility affecting the level of expression of *Shh* pathway factors is the hypothesized concept of cancer stem cells which have the capacity of self-renewal and unlimited replication (29-31). Bailey *et al* also identified the so-called cancer stem cell of the pancreas (32) and Tian *et al* studied lung cancer and observed that the *Shh* pathway is activated mainly in the cancer stem cells and not in every cancer cell (23). The effect of cyclopamine upon the *Shh* pathway may have occurred only in the cancer stem cells of our HCC mice and not in all cancer cells. Cyclopamine may affect the mRNA expression. The key target factor of the *Shh* pathway in the inhibition of cancer remains controversial (24,33,34). Interference with *Shh-Gli-1* signaling may inhibit the proliferation of prostate cancer cells (35). Chen *et al* considered that the downregulation of Bcl-2 was important in HCC following cyclopamine treatment (19). Kim *et al* reported that the suppression of *Gli-2* expression is significant (25).

There are some limitations of the present study. One is that the most effective and tolerable dose of cyclopamine for the treatment of HCC in mice requires further study. The second is that the side-effects of this drug at higher doses in humans need to be understood. The third is that it remains unknown whether the treatment outcome would be improved if a longer treatment period was used.

We conclude that cyclopamine may effectively inhibit HCC in mice *in vivo*. The results also indicate that blockade of the *Shh* signaling pathway may potentially be an effective treatment target for HCC.

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