

Activation of sonic hedgehog signaling pathway is an independent potential prognosis predictor in human hepatocellular carcinoma patients

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Objective: The activation of hedgehog (HH) pathway is implicated in the development of human malignancies including hepatocellular carcinoma (HCC). However, the clinical impact of HH activation in HCC patients is still unclear. This study was conducted to confirm whether the expression of HH pathway components was associated with HCC progression and clinical outcome.

Methods: This study was a sample-expanded and prolonged follow up of one of our previous studies. It included 46 HCC patients who underwent surgical treatment from 2002 to 2005. The expression of sonic HH (*SHH*), patched-1 (*PTCH1*), smoothened (*SMOH*) and glioma-associated oncogene-1 (*GLI1*) genes in tumor and adjacent normal tissues extracted from the patients were examined by reverse transcription-polymerase chain reaction (RT-PCR) to explore the relationship between these genes and the clinical prognosis of HCC.

Results: The expression levels of *SHH*, *PTCH1*, *SMOH* and *GLI1* in HCC tissues were 60.87%, 50.00%, 32.61% and 54.35%, respectively. The expression levels of *SHH*-related molecules were relatively intense in cancer tissue, but insignificantly correlated with any clinicopathological factors of tumor. Transcriptional factor *GLI1* was the only molecule associated with poor prognosis among the HCC patients. The expression of *GLI1* gene in tumor tissues was significantly related with disease-free survival (DFS) ($P=0.042$) and overall survival (OS) ($P=0.030$). The simultaneous expression of *GLI1* in tumor and adjacent normal liver tissues correlated with DFS ($P<0.029$) and OS ($P<0.025$).

Conclusions: HH signaling activation is an important event in the development of human HCC. The expression of *GLI1* in SHH pathway is possibly involved in HCC progression, which may be a useful prognostic indicator of HCC.

Key Words: Hepatocellular carcinoma; hedgehog; *GLI1*; sonic hedgehog; patched; smoothened; prognosis



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Introduction

Liver cancer, especially hepatocellular carcinoma (HCC), is a malignancy of worldwide significance (1,2). Although the increased global incidence of HCC is correlated with the increasing prevalence of chronic infection with hepatitis B virus (HBV) and hepatitis C virus (HCV) (2,3), some of the

mechanisms associated with the initiation and progression of this disease remain elusive.

Dysregulation of the hedgehog (HH) pathway is implicated in the carcinogenesis of multiple tissue types (1,4). HH was first identified in *Drosophila* during screening of genes that are important in early embryonic development (5). This pathway

is activated during binding of sonic HH (*SHH*) or Indian hedgehog (*IHH*) ligand to their receptors, Patched (*PTCH*). The unbound *PTCH* acts as a tumor suppressor that can bind to and repress smoothened (*SMOH*), thereby preventing the *SMOH* proto-oncoprotein from activating downstream of the transcription factors, such as glioma-associated oncogene-1 (*GLI1*). By contrast, the ligand-bound *PTCH* facilitates the release of *SMOH* and activation of *GLI1* resulting in the transcription of target genes including *PTCH* and *GLI1* (1).

The HH activation has been observed in other types cancers such as basal cell carcinomas of the skin (6-9), prostate cancer (10,11), lung cancer (12,13), gastrointestinal cancers (14-19), breast cancer (20,21), and ovarian cancer (22). Although, it is required in liver embryogenesis (14,23), the HH signaling pathway is not well-sustained in the adult liver because of the insufficient HH pathway activity of mature hepatocytes (1,23). Recent studies showed that the HH pathway is frequently activated in HCC (1-3). The increased expression of *GLI1* protein in breast tumor and hepatoblastoma is also reportedly correlated with significantly poor prognosis (24-26). The HH pathway mediates the progression of breast cancer from non-invasive to invasive and serves as a significant independent prognostic indicator in gastric and bladder cancers (27,28).

In our previous studies, we found an association with poor clinical prognosis of the HH pathway in human HCC (29) as well as the simultaneous expression of *GLI1* in HCC and liver tissues adjacent to the tumor. These findings prompted us to expand our sample size to 46 HCC patients and extend their follow ups to confirm whether the expression of HH pathway components is associated with HCC progression and clinical outcome.

Materials and methods

Patients and tumor samples

This study included 46 HCC patients consisting of 40 (86.96%) males and 6 (13.04%) females with ages ranging from 35 years to 79 years (median age =49 years). All patients underwent surgical treatment from April 2002 to July 2005 in Beijing Cancer Hospital. The clinical and pathologic data included patients' demographics (age, gender), tumor size, degree of histological differentiation, and complete follow-up record. The exclusion criterion was preoperative therapy. The study was approved by the Ethics Committee of Human Experimentation of Beijing Cancer Hospital.

All samples of tumor and adjacent normal liver tissues were freshly obtained immediately after surgery. The

samples of tumor tissue were collected from the luminal aspect of the malignancy, whereas those of paired adjacent normal tissue were from the luminal aspect of the liver tissues 2 cm away from the tumor margin. All tissue samples were snap-frozen in liquid nitrogen for 30 min after resection and stored at -80 °C.

RNA extraction and complementary DNA (cDNA) preparation

The total RNA was isolated from 100 mg of each tissue sample using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions, and stored at -80 °C for further use. The reverse transcription (RT) of the total RNA (1 µL) was performed using SuperScript First-Strand Synthesis System (Invitrogen). The internal control was glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; forward primer: 5'-TCA ACG GAT TTG GTC GTA TT-3', reverse primer: 5'-AGT CTT CTG GGT GGG AGT GAT-3', 540 bp).

Polymerase chain reaction (PCR) amplification

To analyze the expression of individual HH gene, 1 µL of cDNA was amplified with 6.25 units of AmpliTaq Gold (Roche, Basel, Switzerland) in 25 µL reaction solution containing 0.5 mmol/L dNTPs and 1.5 mmol/L MgCl₂. The primer sequences for PCR of each gene were designed according to a previous study (9) or using Genbank sequences (Table 1). All reactions were carried out in a PTC-100 Peltier Thermal Cycler (MJ Research, Waltham, MA, USA). Electrophoresis was performed by loading 8 µl of each sample on a 1% agarose gel. The reaction result was visualized by ethidium bromide staining using the Bio-imaging System (Ultra-Violet Products, UVP, Cambridge, UK).

DNA sequencing

The representative PCR products of each gene were measured by Beijing AuGCT Biotechnology Co. Ltd. and were screened using Chromas 2.3 shareware (Technelysium, Australia).

Western blot analysis

The total proteins were extracted from the prepared samples of fresh HCC and corresponding adjacent normal liver tissues, and the concentration was measured by bicinchoninic acid (BCA) protein assay. The protein sample

Table 1 Primers and fragment sizes of HH signaling genes

Genes	Primers	Fragment sizes (bp)
<i>SHH</i>	F: 5'-CCA CTG CTC GGT GAA AGC AG-3' R: 5'-GGA AAG TGA GGA AGT CGC TG-3'	181 (nt 694-875)
<i>PTCH1</i>	F: 5'-CGC-CTA TGC CTG TCT AAC CAT GC-3' R: 5'-TAA ATC CAT GCT GAG AAT TGC A-3'	450 (nt 1,338-1,788)
<i>SMOH</i>	F: 5'-CAC CTC CAA TGA GAC TCT GTC C-3' R: 5'-CTC AGC CTG GTT GAA GAA GTC G-3'	519 (nt 918-1,437)
<i>GLI1</i>	F: 5' CTC AAC AGG AGC TAC TGT GG-3' R: 5'-GGG TTA CAT ACC TGT CCT TC-3	396 (nt 2,789-3,185)

F, forward primer; R, reverse primer

Table 2 Summary of *SHH*, *PTCH1*, *SMOH* and *GLI1* expression in HCC and adjacent liver tissues by RT-PCR

Indices	Rate (% , N=46)	P value of rate	Mean*	P value of mean
<i>SHH</i>				
Cancer tissue	60.87 (28/46)	0.052	35.02±51.28	0.001
Adjacent normal tissue	39.13 (18/46)		11.91±30.44	
<i>PTCH1</i>				
Cancer tissue	50.00 (23/46)	NS	53.33±93.03	0.179
Adjacent normal tissue	45.65 (21/46)		33.96±90.06	
<i>SMOH</i>				
Cancer tissue	32.61 (15/46)	0.045	13.20±24.59	0.002
Adjacent normal tissue	13.04 (6/46)		1.95±5.53	
<i>GLI1</i>				
Cancer tissue	54.35 (25/46)	NS	50.51±88.45	0.160
Adjacent normal tissue	34.78 (16/46)		33.56±61.07	

NS, not significant; *Analyzed by Bio-imaging System (Ultra-Violet Products, UVP, Cambridge, UK)

(70 µg) was separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, and then transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford). The primary antibodies were rabbit anti-human SHH polyclonal antibody (Santa Cruz Biotechnology, sc-9024) and anti-GAPDH antibody (Zhongshan Goldenbridge Biotechnology, Ta-08). The secondary antibodies were anti-rabbit IgGs conjugated to horseradish peroxidase (HRP). The blots were developed with the Pico West illumination kit (Promega, Wisconsin, USA). The results were compared by ImageJ (NIH, Maryland, USA) and the ratio of protein gray levels of SHH/GAPDH was calculated.

Statistical analysis

The disease-free survival (DFS) is the time from initial diagnosis to relapse or metastasis. The overall survival (OS) is the time from initial diagnosis to death due to any cause or the date of last follow up. Statistical data were analyzed using the SPSS software (SPSS Inc., Chicago, IL, USA).

The pairwise correlation between the continuous clinical outcomes and target gene expression levels were estimated using Spearman's rank correlation (P). P<0.05 (two tailed) was considered statistically significant. The survival time distribution was estimated using the Kaplan-Meier method.

Results

Expression of individual SHH gene

We detected the expression of HH signaling molecules in 46 paired normal liver and HCC samples by RT-PCR. In the HCC samples, *SMOH* was detected in 15 (32.61%) samples, *PTCH1* in 23 samples (50.00%), *SHH* in 28 samples (60.87%), and *GLI1* in 25 (54.35%) samples. No significant difference was observed between the expression levels of *PTCH1* and *GLI1* in the normal liver and tumor tissues, whereas the overexpression of *SHH* was observed in HCC samples (P=0.001). The expression of the signaling transmembrane protein gene *SMOH* was significantly increased in HCC samples (P=0.002) (Figure 1, Table 2).

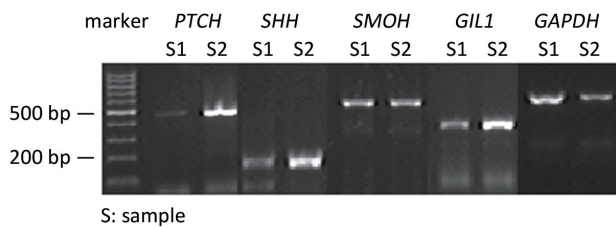


Figure 1 Expression of *SHH*, *PTCH1*, *SMOH* and *GLI1* genes in HCC samples

Expression of individual SHH protein

The molecular weight of SHH protein was about 27 kD. The GAPDH protein expression levels were stable in all tissues samples. SHH protein expression was detected in 12 cases of HCC and corresponding adjacent normal liver tissues, whose *SHH* mRNA was positively expressed in liver tumor tissues. We found that SHH protein was significantly positively expressed in human HCC tissues but negatively or weakly expressed in adjacent normal liver tissues. However, in 1 case of severe cirrhotic adjacent non-tumor liver tissue, SHH protein was strongly positively expressed (Figure 2). This was consistent with the result of our previous immunohistochemical detection (29).

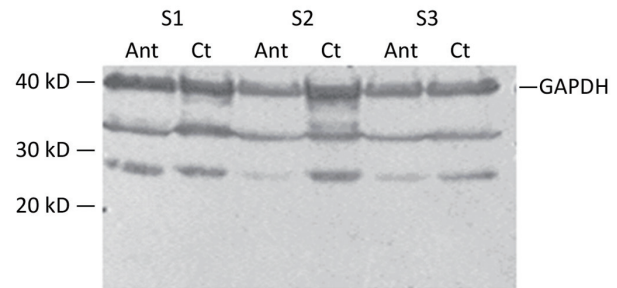
Correlation between expression of SHH signaling genes and clinical prognosis of HCC

All 46 enrolled patients had a complete follow-up record. The median follow-up time was 30 months (range: 1-83 months).

We found no significant relationship between the expression levels of SHH signaling genes (except *GLI1*) in tumor tissues and clinical prognosis in the 46 enrolled HCC patients. The expression of transcriptional factor *GLI1* in tumor tissues showed a significant relationship with DFS ($P=0.042$) and OS ($P=0.030$) (Figure 3A,B). The co-overexpression of *SHH* and *GLI1* genes in tumor tissues showed a significant relationship with DFS ($P=0.024$) and a trend of influence on the OS of 46 HCC patients ($P=0.083$) (Figures 3C,D).

For adjacent non-tumor liver tissues, we found the expression levels of *SHH*, *SMOH* and *PTCH1* did not show a significant relationship with the clinical prognosis, while the expression of *GLI1* showed a significant relationship with DFS and OS ($P<0.05$). The 4-year DFS rates of patients whose *GLI1* expressed positively and negatively in adjacent liver tissues were 8.9% and 50.4%, respectively ($P=0.041$). The 5-year OS rates were 21.4% and 34.7%, respectively ($P=0.042$) (Figure 3E,F).

The co-overexpression of *GLI1* gene in tumor tissues



S: sample; Ct: cancer tissue; Ant: adjacent non-tumor tissue

Figure 2 Western blot analysis for SHH. S1. SHH protein expression is positive in some cirrhotic adjacent non-tumor liver tissues; S2 and S3. SHH protein expression is significantly positive in human HCC tissues and negative or weakly observed in adjacent non-tumor liver tissues

and adjacent liver tissues was significantly associated with clinical prognosis ($P<0.05$). Compared with patients whose *GLI1* gene was not simultaneously positively expressed in tumor tissues and adjacent liver tissues, those patients with *GLI1* co-overexpression had 2-year DFS rates of 21.2% and 57.3%, respectively ($P=0.029$), as well as 5-year OS rates of 18.5% and 42%, respectively ($P=0.025$) (Figure 3G,H).

Correlation between expression of SHH signaling genes and patients' characteristics

The patients' characteristics are listed in Table 3, and the relationship between gene expression patterns and patients' characteristics were analyzed. Overall, the 46 tumor samples increased in *SMOH* proto-oncogene expression (mean: 13.20 ± 24.59). *SMOH* expression was up-regulated in 15 HCC samples (32.61%). Furthermore, *SMOH* proto-oncogene expression in tumor positively correlated with the HCC tumor size ($\rho=0.306$, $P=0.041$) (Figure 4A). We also found that the *GLI1* mRNA transcript levels had a trend of correlating with the HCC tumor size ($\rho=0.277$, $P>0.065$), whereas the *SHH* mRNA transcript levels had no correlation with the size of liver tumor ($P>0.20$).

The serum levels of tumor marker α -fetoprotein (AFP) are clinically used in the diagnosis and follow-up of patients with malignant liver tumors. Therefore, we examined the relationships among preoperative serum AFP levels, tumor size, as well as the expression of *PTCH* tumor-suppressor gene and *SMOH* proto-oncogene. In our cohort study, the serum AFP levels were elevated in 35 cases (76.09%). However, no empirical evidence suggested that the tumor size correlated with the preoperative serum AFP level

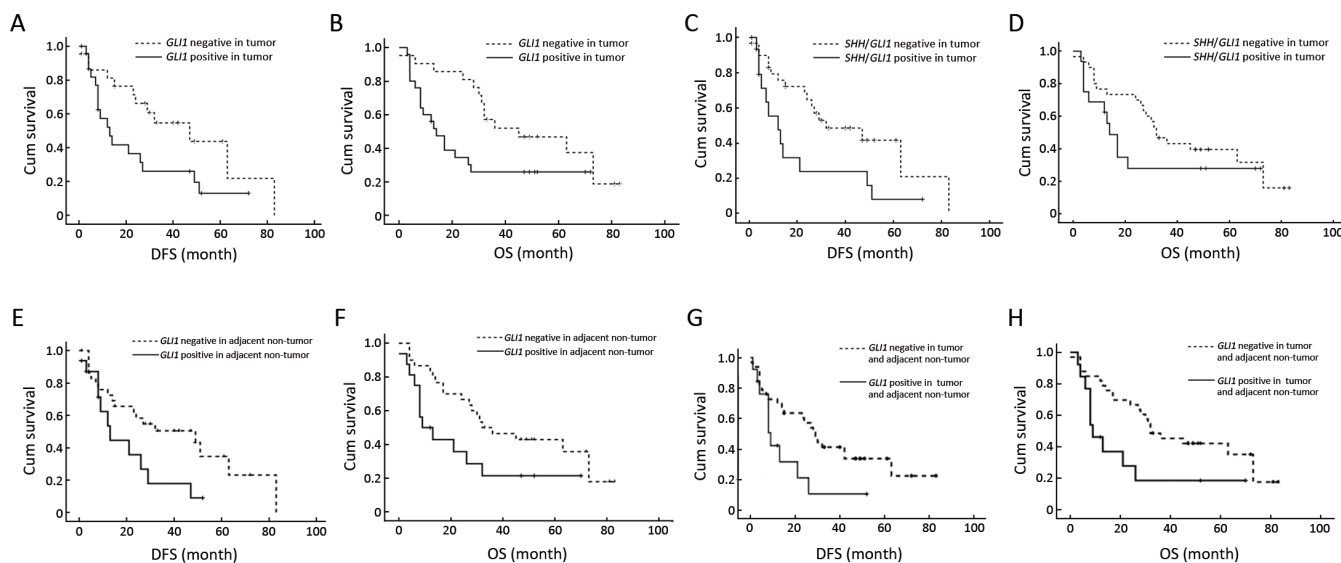


Figure 3 The expression of *GLI1* and *SHH* in tumor tissues and adjacent non-tumor liver tissues was correlated with DFS and OS. The expression of *GLI1* in tumor tissues was correlated with DFS (A, $P=0.042$) and OS (B, $P=0.030$) of 46 HCC patients. The expression of *GLI1* and *SHH* in tumor tissues was correlated with DFS (C, $P=0.024$) and OS (D, $P=0.083$) of the patients. *GLI1* expression in adjacent non-tumor liver tissues was correlated with DFS (E, $P=0.041$) and OS (F, $P=0.042$) of the patients. *GLI1* expression in tumor tissues and adjacent non-tumor liver tissues was correlated with DFS (G, $P<0.029$) and OS (H, $P<0.025$) of the patients

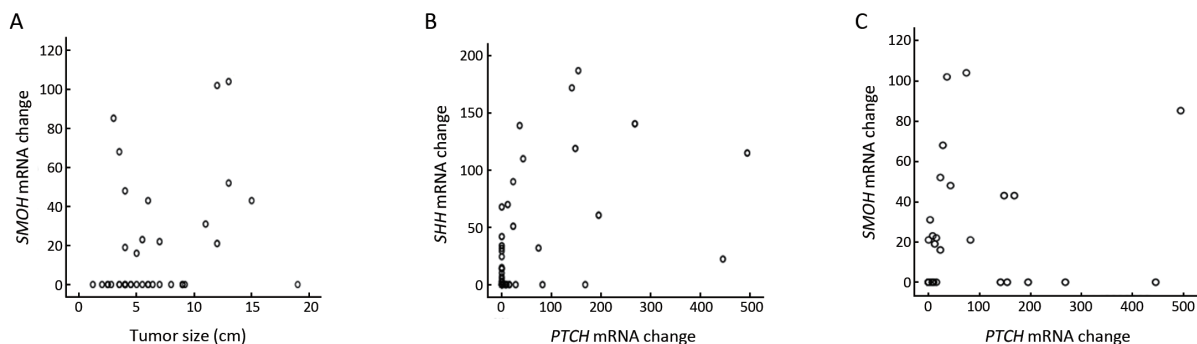


Figure 4 Correlation among *SHH* component gene expressions in HCC and matched non-tumor liver tissues. A. *SMOH* mRNA expression was correlated with the tumor size ($\rho=0.306$, $P=0.041$); B. *SHH* expression was correlated with *PTCH* in tumor tissues ($\rho=0.381$, $P=0.009$); C. *SMOH* expression was correlated with *PTCH* in tumor tissues. ($\rho=0.558$, $P<0.001$)

($\rho=0.193$, $P=0.325$). The serum AFP levels also had no correlation with the expression levels of *SHH*, *PTCH*, *SMOH* or *GLI1* gene in the tumor samples ($P>0.30$). But interestingly, the serum AFP level inversely correlated with DFS ($\rho=-0.483$, $P=0.009$) and OS ($\rho=-0.390$, $P=0.040$). Moreover the tumor size also inversely correlated with DFS ($\rho=-0.131$, $P=0.389$) and OS ($\rho=-0.189$, $P=0.214$) although the relationship was not statistically significant.

We found that HCC with *PTCH* overexpression had significantly higher *SHH* ($\rho=0.381$, $P=0.009$) (Figure 4B) and *SMOH* expressions ($\rho=0.558$, $P<0.001$) (Figure 4C).

Moreover, HCC with *PTCH* overexpression in adjacent non-tumor liver tissues also tended to have higher *SMOH* ($\rho=0.485$, $P=0.001$) and *SHH* expressions ($\rho=0.359$, $P=0.015$) in tumor tissues. This result suggested that *SHH* overexpression in some of the tumors was associated with increased HH activity.

Discussion

The *HH* gene family, which codes a much more sophisticated set of secreted proteins, was first identified

Table 3 Demographics, underlying diseases and tumor related factors for the cohort study

Indices	Case (N=46)	Percentage (%)
Gender		
Female	6	13.04
Male	40	86.96
Age (year)		
Mean	51.67±11.18	
Median	49	
Range	35-79	
Underlying disease		
Yes	45	97.78
No	1	2.17
Viral	45	97.78
HBV	43	93.47
HCV	3	6.52
Liver cirrhosis	35	76.09
Cryptogenic	1	2.17
Serum AFP (ng/mL)		
Normal	11	23.91
High	35	76.09
Mean	14,606.22±49,814.14	
Median	930.20	
Range	3.62-220,300.00	
Tumor size (cm)		
Mean	6.30±3.86	
Median	5.0	
Range	1.2-19.0	

in *Drosophila* in 1980 (5) and essential for early embryo patterning. Previous studies have reviewed that the HH signaling pathway plays key roles in various processes, such as embryogenesis, maintenance of adult tissue homeostasis, tissue repair during chronic persistent inflammation, and carcinogenesis (3,30,31). SHH is active only in stem cells and/or endodermal progenitor in adults (16,23). Recent studies showed that aberrant signaling of this pathway is involved in a variety of human cancers (6-20,21-32).

The HH pathway essentially consists of PTCH, the motive protein, SMOH, and the transcriptional factors GLI2 and GLI3. GLI1 is induced only upon activation of the HH pathway (24,26). Therefore, as a transcription factor, GLI1 expression is a good indicator of HH pathway activation. Recent studies showed that Gli1 controls several biological characteristics, such as proliferation and invasion, in several types of cancers (17,19,26).

Although the activation of the HH pathway is involved in several types of gastrointestinal cancers and other cancers, its role in HCC pathogenesis is not well understood. The normal hepatocytes lack the HH signaling pathway (1,23). However, the activation of the HH pathway in endodermal

progenitors is essential for liver development. Thus, we hypothesized that regulation of the HH signaling may be involved in hepatocarcinogenesis.

Our data indicated that HH signaling is frequently activated in HCC. *SHH* and its target genes, *PTCH1*, *SMOH* and *GLI1*, were frequently expressed in the tumor tissues than in the adjacent liver tissues. These data support our hypothesis that activation of the HH pathway is essential in the development of HCC. Since the HH signaling pathway is frequently activated in HCC, the markers for the activation, including *SHH*, *SMOH*, *PTCH1* and *GLI1*, may be useful for diagnosis of liver cancers.

Sicklick (1) reported that the expression of *SMOH* proto-oncogene is positively correlated with HCC tumor size. Our results also showed that overexpression of *SMOH* mRNA in HCC was positively correlated with HCC tumor size. Thus, it can be a prognostic indicator in HCC biology. Although the serum AFP level was inversely correlated with DFS and OS, it was not related to the expression of SHH pathway genes. Moreover, the tumor size was also inversely correlated with DFS and OS, but with no statistically significant relationship. This data showed that *SMOH* activation is a potential prognostic indicator of human HCC. Ten Haaf, *et al.* (24) found that the increased expression of GLI1 protein in breast cancer is significantly correlated with unfavorable OS. Souzaki, *et al.* (26) reported that the %GLI1 nuclear translocation in lymph nodes with micro-metastasis was higher than that in ductal carcinoma in situ (DCIS) with microinvasion and DCIS. The progression from DCIS to invasive ductal carcinoma (IDC) requires a certain level of %GLI1 nuclear translocation and the HH pathway contributes to the progression from DCIS. He, *et al.* (28) also reported that patients with positive expression of SHH, PTCH1 and GLI1 proteins showed poorer DFS and OS than those with negative expression, and these proteins were independent, unfavorable prognostic factors.

In this study, we found that *GLI1* expression in HCC tissues showed a significant relationship with DFS and OS. The simultaneous positive expression of *GLI1* gene in tumor and adjacent non-tumor liver tissues was significantly related with clinical prognosis. These data suggest that the activation of SHH signaling pathway is potential prognostic indicator in human HCC. The markers for HH signaling activation, especially *GLI1*, may be useful for the judgment of clinical prognosis.

In general, the enhanced HH pathway activation leads to downstream expression of target genes, including *PTCH* and *GLI1*. Thus, the levels of these transcripts are often

used as surrogate markers of HH pathway activity (33). However, recent studies suggested that other less-understood mechanisms also influence the levels of *PTCH* and *GLI1* transcripts. HCC often develops in cirrhotic livers (34,35) and others have demonstrated *PTCH* transcripts in some cirrhotic patients (36,37). Increased *SMOH* mRNA levels are also observed in some cirrhotic patients (36). Up to three-fourth (35/46) of the HCC patients in our study had underlying cirrhosis. Therefore, the interindividual differences in *PTCH* expression in non-neoplastic liver tissues also influence our results.

In our study, *SHH* overexpression resulted in higher *PTCH* expression. Thus, the HCC with higher *PTCH* levels tends to have higher *SMOH* expression. This suggests that dysregulation of HH signaling occurs during hepatocarcinogenesis and likely resulted from increased *SMOH* that occurs without the accompanying increase in *PTCH* expression, which is typically observed in other gastrointestinal tumors (14,15). This observation was consistent with other previous studies (1,3,4).

Several studies have demonstrated that SHH signaling pathway not only plays a critical role in the development of human gastrointestinal tract, but also has a close relationship with gastrointestinal adenocarcinoma and precancerosis (32,38), while others have found that *SHH* gene expression was higher during the early disease stage during which more undifferentiated cells were found, than in advanced disease stage (3,15,20,38). HH signaling pathway is also reportedly activated during the fibroproliferative response to chronic cholestatic biliary injury in primary biliary cirrhosis (39). These suggested that HH signaling pathway has an early and critical role in carcinogenesis (20).

Huang, *et al.* (3) 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 HCC, whereas a noncancerous liver tissue did not have any detectable expression of *SHH*, *PTCH1* and *GLI1*. This indicated that HH signaling activation occurs in early lesions of HCC. Our data showed active SHH signaling genes in adjacent liver tissues of HCC, especially in some cirrhotic patients. The overexpression of *GLI1* gene in adjacent liver tissues suggests a worse prognosis. This provided evidence that HH signaling may play a previously unsuspected role in the progression from cirrhosis to liver cancer. Although the pathological examination found no cancer cells existed in the tissue samples expressing *GLI1* gene, the possibility of the tumor-induced abnormal cell differentiation in these tissue cannot be ruled out. The SHH pathway genes were

not only overexpressed in malignant tumors, but also play a multifaceted role. The HH pathway activation may occur as an early event during the evolution of hepatic neoplasm. Overall, these data support that tumorigenesis occurs is an injury/repair-related process, and suggest that dysregulation of the HH pathway contributes to liver regeneration, namely liver cancer.

Sicklick (1) reported the missense mutation of oncogenic *SMOH* gene in HCC. This does not rule out the possibility that other unidentified *SMOH* mutations may exist in HCC and that HH signaling may play a previously unsuspected role in the progression from cirrhosis to liver cancer. Thus, the mutation statuses of *PTCH*, *SMOH*, *GLI1* and other signaling molecules in HCC requires further analysis to determine whether the mutations of these genes may be present during HCC development.

Several studies have indicated that the HH pathway may be a potent therapeutic target for tumors including HCC. A series of studies with a HH pathway inhibitor, cyclopamine, has brought about this expectation. Cyclopamine was discovered through epidemiological investigations of malformed sheep (40). Cyclopamine can reportedly inhibit HH ligand-dependent and independent HH pathway activation through direct interaction with *SMOH* (41-44). The HH pathway may be a potential therapeutic target in HCC.

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