Hepatocellular carcinoma (HCC) is one of the most common cancers in Asia and Africa, and its incidence is increasing worldwide (1). Despite recent advances in early diagnosis and treatment, the prognosis for HCC remains very poor. Most HCC cases arise in the setting of chronic hepatitis virus infection. Alcohol consumption, dietary aflatoxin, and exposure to chemical carcinogens are also implicated in its pathogenesis (1, 2). Although the etiologic factors involved in HCC are well known, the genetic events underlying its carcinogenesis are still unclear.

The Hedgehog (Hh) pathway is indispensable for human development and tissue polarity (3). The binding of secreted Sonic Hh (SHH) and Indian Hh to their receptor, patched (PTCH), which represses the activity of the transmembrane protein smoothened (SMO) in the absence of ligand, leads to alleviation of PTCH-mediated suppression of SMO (4). Derepression of SMO results in the activation of downstream targets through the activation of the transcription factor, glioma-associated oncogene homologue (GLI; ref. 4). Two types of aberrant activation of Hh signaling are involved in carcinogenesis: ligand-independent and ligand-dependent activation. The former is due to oncogenic mutation in signal components, such as SMO and PTCH, and has been reported in sporadic basal cell carcinoma, medulloblastoma, and Gorlin syndrome, which is characterized by numerous basal cell carcinoma, rhabdomyosarcoma, and medulloblastoma (5–9). Ligand-dependent activation with Hh overexpression has been reported in gastric, pancreatic, prostate, and small cell lung cancers (10–14).

Recently, the aberrant activation of Hh signaling in HCC has also been reported (15–17). The authors showed that the overexpression of SMO or induced expression of SHH was the

**Abstract**

**Purpose:** Hedgehog (Hh) signaling is activated in several cancers. However, the mechanisms of Hh signaling activation in hepatocellular carcinoma (HCC) have not been fully elucidated. We analyzed the involvement of Hh-interacting protein (HHIP) gene, a negative regulator of Hh signaling, in HCC.

**Experimental Design:** Glioma-associated oncogene homologue (Gli) reporter assay, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay, and quantitative real-time reverse transcription – PCR for the target genes of the Hh signals were performed in HHIP stably expressing hepatoma cells. Quantitative real-time PCR for HHIP was performed in hepatoma cells and 36 HCC tissues. The methylation status of hepatoma cells and HCC tissues was also analyzed by sodium bisulfite sequencing, demethylation assay, and quantitative real-time methylation-specific PCR. Loss of heterozygosity (LOH) analysis was also performed in HCC tissues.

**Results:** HHIP overexpression induced significant reductions of Gli reporter activity, cell viability, and transcription of the target genes of the Hh signals. HHIP was hypermethylated and transcriptionally down-regulated in a subset of hepatoma cells. Treatment with a demethylating agent led to the HHIP DNA demethylation and restoration of HHIP transcription. HHIP transcription was also down-regulated in the majority of HCC tissues, and more than half of HCC tissues exhibited HHIP hypermethylation. The HHIP transcription level in HHIP-methylated HCC tissues was significantly lower than in HHIP-unmethylated HCC tissues. More than 30% of HCC tissues showed LOH at the HHIP locus.

**Conclusions:** The down-regulation of HHIP transcription is due to DNA hypermethylation and/or LOH, and Hh signal activation through the inactivation of HHIP may be implicated in the pathogenesis of human HCC.

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major trigger for Hh signal activation (16, 17). However, the implication of a negative regulator of Hh signaling was not fully investigated. On the other hand, Hh signaling regulates angiogenesis, and Hh-interacting protein (HHIP), which functions as a negative regulator of the Hh pathway (18), is down-regulated in endothelial cells during tube formation (19), suggesting that disruption of the HHIP gene may induce vascular-rich tumors, such as HCC. This led us to focus on HHIP. The HHIP gene is expressed in most human fetal and adult tissues (20) and encodes a membrane glycoprotein that binds all three mammalian Hh proteins, i.e., PTCH, Indian Hh, and Desert Hh, with affinity similar to that of PTCCH (21). In the current study, we investigated the involvement of HHIP in hepatoma cell lines and the methylation status, loss of heterozygosity (LOH), and mRNA expression of HHIP gene in 36 HCC cases.

Materials and Methods

Cell culture. The human hepatoma and hepatoblastoma cell lines HLE, HuH7, HepG2, HuH6, and PLC/PRF/5 were obtained from the Health Science Resources Bank, and Hep3B was obtained from the Cell Resource Center for Biomedical Research. The cell lines were grown in DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin.

Patients. Liver tissues were obtained from 36 HCC patients (31 men and 5 women; mean age, 62.3 ± 11.0 y) who underwent surgical resection at Chiba University Hospital. Among these patients, six were positive for hepatitis B surface antigen and 25 were positive for hepatitis C virus antibody, whereas the remaining five patients lacked evidence of either viral infection. Based on the histologic findings, the 36 HCC tumors were classified as follows: 6 well-differentiated, 25 moderately differentiated, and 5 poorly differentiated tumors. More than 50% of the tumors developed in cirrhotic livers (Supplementary Table S1). HCC samples and corresponding nontumor liver tissues obtained by surgical resection were immediately frozen at -80°C. All patients gave informed consent for their participation, and the ethics committee approved these studies.

Establishment of cells with stable expression of HHIP. We purchased the full-length human HHIP clone that encodes the open reading frame of HHIP from GeneCopeia and inserted it into the pCMV-Tag4 mammalian expression vector (Stratagene), thereby generating the pCMV-Tag4-HTIP vector. After confirming the insertion by sequencing, we transfected the pCMV-Tag4-HTIP vector into the Hep3B cells, which were confirmed as HHIP-null cells by quantitative real-time reverse transcription–PCR (RT-PCR) and Western blotting (Fig. 1; data not shown). To establish cell lines that stably express HHIP, we cultured the transfected cells in the presence of 80 μg/ml Genetecin (Wako). Genetecin-resistant colonies appeared within 2 wk, after which the cells were expanded for another 3 wk to produce the original stock cells. Four HHIP stable transfectants (H7-HHIP1, H7-HHIP2, and 3B-HHIP1, 3B-HHIP2) and two control transfectants (H7-MOCK and 3B-MOCK) were selected for further study.

Western blotting. Western blotting was done using proteins of total fractions and the primary antibodies for rabbit anti-SHH (1:250; Santa Cruz Biotechnology), rabbit anti-GLI1 (1:1,000; Cell Signaling Technology), rabbit anti-Hip (R&D Systems), mouse anti-β-actin (ACTB; 1:10,000; Sigma-Aldrich), and mouse anti-FLAG M2 (1:1,000; Sigma-Aldrich). The appropriate horseradish peroxidase–conjugated antibodies (1:1,000; Amersham) were used as secondary antibodies. An enhanced chemiluminescence detection system (ECL-plus, Amersham) was used for detection. Experimental procedures were performed as previously described (10).

DNA Hypermethylation and LOH of HHIP in HCC

Gli reporter assay. Hepatoma and hepatoblastoma cell lines, HHIP stably transfected cells, and the control MOCK cells, were seeded into 12-well plates and grown to ~ 50% to 70% confluence for 24 h before being transiently transfected with 0.5 μg of 8′ × 3′Gli-Bs-Δ51 Luc II, a gift from Dr. Hiroshi Sasaki (Center for Developmental Biology, RIKEN; ref. 22) and 50 ng of pRL-SV40 vector (Toyo Ink) using Fugene 6. This Gli-luciferase reporter construct contains eight copies of the consensus Gli-binding site upstream of a 6-crystallin basal promoter (22, 23). Cells were harvested 48 h after transfection, and luciferase assays were performed with the Picagene Dual Sea Pansy system (Toyo Ink). Firefly and sea pansy luciferase activities were measured as relative light units using a luminometer (Lumat LB 9507, EG&G Berthold). All assays were performed at least in triplicate.

Cyclopamine treatment. All six hepatoma and hepatoblastoma cell lines were seeded into six-well plates and grown in medium containing the pharmacologic Hh inhibitors, cyclopamine (Toronto Research Chemicals) at concentrations of 0 to 10 μmol/L for 96 h. We changed the medium every 2 d.

3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay. The numbers of viable cells were determined using a CellTiter 96 AQone One Solution Cell Proliferation Assay (Promega) according to the manufacturer’s instructions. In brief, cells were plated on six-well tissue culture plates at a density of 3.0 × 104 per well. Ninety-six hours after seeding, the cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent solution for 2 h at 37°C. The absorbance at 490 nm was recorded using an ELISA plate reader. All assays were performed at least in triplicate.

5-Aza-2′-deoxycytidine treatment. For demethylation experiments, all six hepatoma and hepatoblastoma cells were plated at a density of 5.0 × 105 cells/100-mm dish and cultured for 24 h, followed by 96 h of culturing with 1 μmol/L 5-aza-2′-deoxycytidine (5-aza-CdR; Sigma-Aldrich).

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Quantitative real-time RT-PCR. Three micrograms of total RNA from each sample were subjected to RT reaction using random oligonucleotide primers and Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Real-time PCR for HHIP, PTCH, and GLI1 genes was performed using the cDNAs of six hepatoma and hepatoblastoma cell lines untreated or treated with 5-aza-CdR, HCC tissues, and corresponding nontumor tissues. Quantitative real-time PCR was also performed for PTCH, GLI1, B-cell lymphoma protein 2 (BCL2), and cyclin D2 (CCND2) genes, the downstream target genes of Hh signaling (24–27), using the cDNAs of the HHIP stably transfected cells and the control MOCK cells. All assays were performed with the TaqMan Gene Expression Assays Inventoried (HHIP, Hs00368450_m1; PTCH, Hs00181117_m1; GLI1, Hs00171790_m1; BCL2, Hs00153350_m1; CCND2, Hs00277041_m1; ACTB, Hs99999903_m1), TaqMan Universal PCR Master Mix, and ABI Prism 7000 Sequence Detection Systems (Applied Biosystems). The standard curve method was used to calculate target gene expression, which was normalized to that of the ACTB gene. Each sample was analyzed in triplicate.

Sodium bisulfite DNA sequencing. Genomic DNA extracted from each of the hepatoma and hepatoblastoma cell lines without 5-aza-CdR treatment was modified by sodium bisulfite using a CpGenome DNA Modification kit (Chemicon International) according to the manufacturer's instructions. The bisulfite-modified DNA was amplified by seminested PCR using the following specific primers: first amplification, forward 5'-TATCC-3'; second amplification, forward 5'-GGTTTAGTATTTCAGGTGGTGGT-3'; and first and second amplifications, reverse 5'-CAACCCACAAATTCTACATTAC-GTATCC-3'. The PCR products were subcloned into the pCR2.1-TOPO vector using a TA cloning kit (Invitrogen) according to the manufacturer's instructions. To determine the CpG methylation status of the 5' CpG island of the HHIP gene, 10 clones from each cell line were sequenced using the ABI PRISM Dye Deoxy Terminator Cycle Sequencing kit and analyzed in an ABI 310 DNA Sequencer (Applied Biosystems). To confirm DNA demethylation by 5-aza-CdR treatment, the genomic DNA from cells treated with 5-aza-CdR was also subjected to bisulfite sequencing when they exhibited DNA hypermethylation of the HHIP gene.

Quantitative methylation specific real-time PCR. Sodium bisulfite-treated genomic DNA samples from the cell lines and liver tissues were analyzed by means of Methylight, a fluorescence-based real-time PCR assay, as described previously (28, 29). Briefly, two sets of primers and probes, designed specifically for bisulfite-converted DNA, were used, a methylated set for HHIP and a reference set for ACTB, to normalize for input DNA. The specificities of the reactions for methylated DNA were confirmed separately using CpGenome Universal Methylated DNA (Chemicon). The percentage of fully methylated molecules at a specific locus was calculated by dividing the HHIP/ACTB ratio of a sample by the HHIP/ACTB ratio of Universal Methylated DNA and multiplying by 100. When the percentage of fully methylated reference defined by the above formula above was ≥4%, the HHIP gene was deemed to be hypermethylated in the sample (29). The primers and probes for HHIP and ACTB were as follows: for HHIP, forward 5'-GTGTAGTCCTGCGG-TAGAGGAGT-3'; reverse 5'-ACAAATATCTACCTT CCGTATAAC-GAA-3'; and probe 5'-FAM-AGTTTAGCGTTGGTTTTG-MGB-3'; for ACTB, forward 5'-TGTGATGAGGAGCTTATGAGT-3', reverse 5'-AACCAATAAACCATCTCCTCCCTTAA-3', and probe 5'-FAM-ACCAC-CACCC AACACAATACAA AACACA-TAMRA-3'. Each sample was analyzed in triplicate.

LOH analysis. LOH was investigated using three polymorphic markers of the 4q31.22 region located within 1.5 Mb of the HHIP locus (centromeric, D4S1604; very close, D4S2998; telomeric, D4S1586). DNA was amplified by fluorescence PCR (30). The primer sequences used were as follows: for D4S1604, forward 5'-TCTGTGCCAGCCAGCAGT-3'; reverse 5'-TTGCAAGCTAGGAGGTTAGTAAGT-3'; for D4S2998, forward 5'-AACGTCTTGGGGCCGACG-3'; reverse 5'-TTCTACACCCAGGC-ACCC-3'; and reverse 5'-CCCGAGTGGCTGATGTGTG-3'. The PCR products were separated by capillary electrophoresis on an ABI 310 Genetic Analyzer using Genescan and GeneMapper 3.7 software (Applied Biosystems). The LOH index was calculated as follows: the peak height of two alleles in each tumor was divided by the peak height in the corresponding nontumor tissues. LOH was defined by an LOH index of <0.67 or >1.5 (31). Allelic deletion of each gene was judged by positive LOH at any of the three sites.

Mutation analysis. Six hepatoma and hepatoblastoma cell lines were subjected to mutation analysis in the 13 exons of the HHIP gene. DNA from these cells was amplified by PCR using the primers designed for the region flanking the intron-exon junction (32). The DNA was sequenced using an ABI PRISM Dye Deoxy Terminator Cycle Sequencing kit and analyzed on an ABI 310 DNA Sequencer (Applied Biosystems).

Statistical analysis. Differences between mean values were evaluated by the unpaired t test, and differences in frequencies were evaluated by Fisher's exact test. Results were considered statistically significant at P < 0.05.
Results

Expression of Hh signaling components and Hh pathway activity in hepatoma and hepatoblastoma cell lines. To analyze whether Hh signaling is present in hepatoma and hepatoblastoma cell lines, we examined the expression of SHH and GLI1 by Western blotting and PTCH expression by quantitative real-time RT-PCR. We also performed Gli reporter assay to investigate Hh pathway activity in these cell lines. The expression of these components and Hh pathway activity were detected in all cell lines to various extents (Supplementary Fig. S1A-C).

HHIP attenuates Hh signaling and induces growth inhibitory effects in hepatoma cell lines. To investigate whether the transfection of HHIP altered the activity of Hh signaling in hepatoma cell lines, we transfected the Gli reporter plasmids into HHIP stably transfected cells and the control MOCK cells (Fig. 2A and B) and measured the Gli reporter activity, an indicator of Hh signaling activity, in these cells. The Gli reporter activities in the HHIP stably transfected cells were reduced by ~50% to ~60%, compared with that in the MOCK cells (P < 0.001; Fig. 2C and D). We also investigated the mRNA expression levels of PTCH, GLI1, BCL2, and CCND2 genes in HHIP stably transfected cells and the control MOCK cells. Compared with the corresponding levels in MOCK cells, the mRNA expression levels of these genes in HHIP stably expressing cells were down-regulated by 30% to 40% (P < 0.01 or 0.05; Supplementary Fig. S2A and B).

Next, to investigate the effect of HHIP on the growth of hepatoma cell lines, the MTS assay was performed on HHIP stably transfected cells. After 96 hours of culture, the viabilities of the HHIP stably transfected cells were ~40% lower than those of the MOCK cells (P < 0.001; Fig. 3A and B).

HHIP transcription is down-regulated through hypermethylation of 5’ CpG islands in hepatoma and hepatoblastoma cell lines. Quantitative real-time RT-PCR analysis revealed that the HHIP transcript was undetectable in HuH7, Hep3B, HepG2, and Huh6 cells without 5-aza-CdR treatment, whereas it was abundant in HLE and PLC/PRF/5 cells (Fig. 1A). Treatment with 5-aza-CdR significantly restored HHIP mRNA expression in HepG2 and HuH6 cells (P < 0.0001; Fig. 1A). The restoration of HHIP protein was also confirmed by Western blotting in HepG2 and HuH6 cells (Fig. 1B). HHIP transcription in HuH7 and Hep3B cells remained undetectable after 5-aza-CdR treatment. The HLE and PLC/PRF/5 cells, with high initial expression of HHIP, exhibited no additional induction of HHIP transcription after 5-aza-CdR treatment (Fig. 1A).

The methylation status of the 46 CpG dinucleotides encompassing the promoter region was examined (Fig. 4A). The 5’ CpG island of HHIP was densely methylated in each HepG2 clone and was partially methylated in Huh6 cells, both of which showed up-regulation of HHIP transcription after 5-aza-CdR treatment, whereas in the HLE, HuH7, Hep3B, and PLC/PRF/5, few of the CpG dinucleotides were methylated. After treatment with 5-aza-CdR, the DNAs of the HepG2 and HuH6 cells were demethylated (Fig. 4B).

Hh signaling was down-regulated after 5-aza-CdR treatment in HepG2 and HuH6 cells. In HepG2 and HuH6 cells, in which HHIP expression increases after demethylation, the mRNA expressions of GLI1 and PTCH were statistically significantly reduced, by ~25% to 30%, after 5-aza-CdR treatment compared with those of 5-aza-CdR–untreated cells (P < 0.01; Supplementary Fig. S3A and B).

Cell lines without expression of HHIP tend to be more sensitive to cycloamine treatment. To explore the relationship of HHIP expression and the sensitivities to cycloamine in hepatoma and hepatoblastoma cell lines, we investigated the viabilities of cycloamine-treated cells by MTS assay. MTS assay revealed that the viabilities of HuH7, Hep3B, HepG2, and Huh6 cells, which have no HHIP expression, were significantly reduced by treatment with >1 μmol/L of cycloamine (P < 0.0001), whereas treatment with 10 μmol/L of cycloamine only led to a significant reduction in cell viabilities in HLE and PLC/PRF/5 cells, in which the HHIP gene was expressed (Supplementary Fig. S4).

Lack of HHIP mutations in hepatoma and hepatoblastoma cells. Six hepatoma and hepatoblastoma cell lines were screened for evidence of mutations in the 13 exons of the HHIP gene. Direct sequencing revealed a complete lack of HHIP mutations in these cell lines.

Fig. 3. Viabilities of HHIP stably expressing cells. A, HuH7 cells. B, Hep3B cells. Points, mean from three independent experiments; bars, SD. * P < 0.001.
HHIP DNA is hypermethylated in HCC tissues. The occurrence of HHIP gene hypermethylation in 36 HCC cases was analyzed using quantitative real-time methylation-specific PCR, Methylight. In the present study, the HHIP gene was defined as being hypermethylated in tissues when the percentage of fully methylated reference value was ≥4. The 5′CpG island of the HHIP gene was shown as being hypermethylated in 21 of 36 tumors (53.6%), whereas, in the corresponding nontumor liver tissues, HHIP hypermethylation was not detected (Table 1). In addition, the percentage of fully methylated reference values of the HHIP gene were significantly higher in the tumors than in the corresponding nontumor liver tissues (5.62 ± 6.39 versus 0.19 ± 0.28, *P* < 0.0001; Fig. 5A). In the 36 HCC patients, no significant correlation was found between HHIP hypermethylation and clinicopathologic characteristics, including sex, age, etiology, state of surrounding liver, differentiation, and size (Supplementary Table S2).

LOH at HHIP gene locus in HCC tissues. DNA samples from 36 HCC tissues were examined for LOH at the HHIP gene locus using fluorescent PCR. At the D4S1604 locus, 26 of 36 cases (72.2%) were heterozygous and 7 of 26 (26.9%) showed LOH. At the D4S2998 locus, 32 of 36 cases (88.9%) were heterozygous and 10 of 32 (31.3%) showed LOH. The heterozygosity of the D4S1586 locus was 23 of 36 (63.9%), and 5 of 23 (21.7%) exhibited LOH (Supplementary Table S3). In total, heterozygosity was achieved in 32 of 36 cases (88.9%), and LOH at the HHIP locus was detected in 10 of 32 cases (31.3%). Of the 10 cases with LOH at the HHIP locus, seven also showed HHIP DNA hypermethylation. No significant correlation was found between HHIP LOH and clinicopathologic characteristics of the 36 HCC patients (Supplementary Table S4).

HHIP mRNA expression is down-regulated in HCC tissues. The relative level of HHIP mRNA was determined by
quantitative real-time RT-PCR as the ratio to the level of ACTB mRNA. In 31 of 36 HCC tissues (86.1%), the level of HHIP transcription was lower than in the corresponding nontumor tissues (Table 1), and HHIP mRNA expression in the HCC tissues was significantly lower than in the corresponding nontumor tissues (3.47 ± 5.84 versus 27.61 ± 34.82, P < 0.0001; Fig. 5B). Of the 31 HCC tissues with down-regulated HHIP transcription, 7 (22.6%) exhibited both HHIP DNA hypermethylation and LOH at the HHIP locus and 24 (77.4%) showed either HHIP DNA hypermethylation or LOH (Supplementary Table S5). There was no case with HHIP DNA hypermethylation or LOH at the HHIP locus that had a higher level of HHIP transcription in the tumor than in the corresponding nontumor tissues. The expression of HHIP mRNA in the tumor was significantly lower in cases with HHIP DNA hypermethylation or LOH at the HHIP locus than in cases showing DNA unmethylation or retention of heterozygosity (0.25 ± 0.47 in hypermethylation cases and 7.97 ± 6.91 in unmethylation cases, P < 0.0001; 0.73 ± 1.19 in LOH cases and 5.27 ± 6.73 in retention of heterozygosity cases, P < 0.05; Supplementary Fig. S5A and B). There was no statistically significant correlation between HHIP transcription and the clinicopathologic characteristics of the 36 HCC patients (Supplementary Fig. S6).

**GLI1 and PTCH mRNA expression in HCC cases.** To assess Hh signaling activation in HCC, we investigated GLI1 and PTCH mRNA expression by quantitative real-time RT-PCR in 36 HCC tissues and corresponding nontumor tissues. We found that GLI1 mRNA transcription was significantly higher in tumors than in corresponding nontumor tissues (1.78 ± 3.77 versus 0.30 ± 0.65, P < 0.05; Supplementary Fig. S7A) whereas PTCH mRNA expression tended to be higher in tumors than in corresponding nontumor tissues, although the difference was not statistically significant (31.11 ± 51.27 versus 12.73 ± 27.79, P = 0.063; Supplementary Fig. S7B). In addition, mRNA expression of GLI1 and PTCH in tumors tended to be higher in the cases with HHIP DNA hypermethylation than in those showing HHIP DNA unmethylation, although the difference was not statistically significant (2.69 ± 4.74 versus 0.50 ± 0.65, P = 0.086, for GLI1, and 43.81 ± 62.15 versus 13.32 ± 21.72, P = 0.078, for PTCH; Supplementary Fig. S8A and B). These results suggested that Hh signaling was activated and that HHIP methylation might be implicated in Hh signal activation in HCC.

**Discussion**

HCC is one of the most frequent human cancers worldwide and has a very poor prognosis (1), despite advances in early diagnosis and therapy. Several studies have indicated that the accumulation of genetic changes occurs in a stepwise manner during the development and progression of HCC, as well as other human cancers. However, the molecular mechanisms underlying the pathogenesis of HCC have not been fully elucidated.

It is well known that aberrant activation of Hh signaling is involved in carcinogenesis. Oncogenic mutation of the Hh pathway has been detected in Gorlin syndrome, sporadic basal cell carcinoma, and medulloblastoma (5–9). On the other hand, overexpression of the ligand in the Hh pathway has been shown to cause pathway activation in gastric, pancreatic, prostate, and small cell lung carcinoma (10–14). It has been reported that the Hh pathway is also activated in HCC (15–17). In our study, as well, Hh signaling components were expressed in hepatoma and hepatoblastoma cell lines to various degrees. Hh signaling plays a major role in multiple aspects of embryonic development, including that of the liver (33), although mature hepatocytes lack Hh signaling activity (11). Therefore, it is possible that the remaining Hh signal-responsive progenitor cells function as cancer stem cells in the liver, leading to the genesis of HCC. The overexpression of SMO or SHH, positive regulators of the Hh pathway, has been shown to be the major trigger for Hh signal activation (16, 17). However, proto-oncogenes are rarely altered in HCC, suggesting that inactivation of tumor suppressor genes is critical for hepatocarcinogenesis (34). Therefore, we focused on the HHIP gene, a

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<td>-</td>
<td>0.011</td>
</tr>
<tr>
<td>35</td>
<td>-</td>
<td>-</td>
<td>0.095</td>
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<tr>
<td>36</td>
<td>-</td>
<td>+</td>
<td>0.084</td>
</tr>
</tbody>
</table>

**Table 1.** DNA methylation, mRNA expression and LOH status of HHIP gene in HCC cases

Abbreviations: T, tumor; NT, nontumor; +, positive; -, negative; NI, not informative.

*When the value of PMR was ≥ 4 in the sample, HHIP DNA was defined as hypermethylated, otherwise HHIP DNA was defined as unmethylated.

†When LOH was detected at any of three markers used in this analysis, LOH status of HHIP gene was defined as positive.
negative regulator of Hh signaling (18), and investigated the involvement of HHIP in HCC.

In the current study, transfection of the full-length HHIP expression vector into two HHIP-null cell lines (HuH7 and Hep3B) led to significant reductions in cell viabilities, Gli-reporter activities, and the PTCH and GLI1 transcripts, which are indicators of Hh signal activation. Transfection of full-length mouse HHIP into mouse testicular epithelial cells (TM3) attenuated their responses to SHH, which shows that HHIP antagonizes Hh signaling when expressed in responding cells (19). Concordant with this, HHIP also attenuated Hh signal activation in hepatoma cells, with consequent growth inhibition. Treatment with cyclopamine, a plant steroidal alkaloid that inhibits the cellular response to the Hh signal (35), also led to reductions in cell viabilities and Hh signal activation in hepatoma cell lines (15–17). Thus, the effect of the over-expression of HHIP is similar to that of cyclopamine on hepatoma cell lines.

In our study, cyclopamine was also observed to cause reduction in cell viabilities. Moreover, interestingly, sensitivities to cyclopamine were higher in HHIP-null cell lines than in HHIP-expressing cell lines. Cyclopamine antagonizes SMO (35), but not HHIP, so the cause of these differences in the sensitivities to cyclopamine in hepatoma cell lines remains to be elucidated. HHIP expression, however, might be useful for the prediction of cyclopamine responsiveness.

To elucidate the involvement of the downstream genes of Hh signaling in HHIP-overexpressed hepatoma cells, we investigated the mRNA expression of BCL2 and CCND2 genes, downstream target genes in the Hh pathway (26, 27). HHIP overexpression resulted in the down-regulation of BCL2 and CCND2 transcription, suggesting that the HHIP-mediated reduction of hepatoma cell viabilities may be due to an increase in apoptosis or cell cycle arrest.

In our study, there was no correlation between intrinsic HHIP expression and Hh signal activation, which was surrogated by GLI1 protein expression, Gli reporter assay, and PTCH mRNA expression. This may be due to other mechanisms for the regulation of GLI1 and PTCH expression.

HHIP expression is decreased in several human tumors of the lung, stomach, colorectal tract, and liver compared with the corresponding normal tissues (15, 19), although HHIP is expressed in most fetal and adult tissues (20). In the present study, HHIP mRNA expression was down-regulated in a subset of hepatoma and hepatoblastoma cell lines, and its expression in the majority of HCC tissues was much lower than in the corresponding non-tumor liver tissues, in concordance with previous reports (15, 19). To elucidate the mechanisms of the down-regulation of HHIP expression, methylation and LOH analyses were performed.

Promoter hypermethylation has recently been identified as a hallmark of human cancer (36). aberrant CpG island hypermethylation is clearly associated with transcriptional silencing of gene expression and plays an important role in the mechanism by which tumor suppressor genes are inactivated in cancer (37, 38). The inactivation through DNA hypermethylation of several tumor suppressor genes, such as E-cadherin, p16INK4a, SOCS, 14-3-3σ, and GSTP1, has also been reported in HCC (39–43).

In the present study, we showed that HHIP mRNA transcription is down-regulated through HHIP DNA hypermethylation in a subset of hepatoma and hepatoblastoma cell lines. Quantitative methylation-specific PCR, Methylight, revealed that HHIP DNA was also hypermethylated in >50% of the HCC tissues, although methylation was not detected in the corresponding non-tumor liver tissues, and the level of HHIP transcription was significantly lower in hypermethylated HCC tissues than that in unmethylated HCC tissues, suggesting that down-regulation of HHIP transcription can be attributed to aberrant hypermethylation of the HHIP gene in HCC. Aberrant methylation of the HHIP gene has also been reported in gastrointestinal and pancreatic cancers (32, 44).

Moreover, the GLI1 and PTCH transcription levels tended to be higher in HHIP-methylated HCC than in HHIP-unmethylated HCC, meaning that the down-regulation of HHIP transcription through HHIP hypermethylation might lead to Hh signal activation in HCC. The reason why the difference in GLI1 and PTCH mRNA expression levels between methylated
DNA Hypermethylation and LOH of HHIP in HCC

HCCs and unmethylated HCCs was not statistically significant could be that there were other regulatory mechanisms for Hh signaling in HCC (16, 17).

HHIP transcription was also down-regulated in HuH7 and Hep3B cells while the HHIP DNA was not methylated and 5-aza-CdR treatment did not lead to restoration of HHIP transcription in these cells. However, genome-wide LOH analysis of hepatoma cell lines using a high-density single-nucleotide polymorphism array and a data analysis tool, Copy Number Analyzer for Affymetrix GeneChip Mapping 100K arrays (45), revealed LOH of the HHIP locus in HuH7 and Hep3B cells. Moreover, because the HHIP gene is located at 4q31.22 and chromosome 4q is frequently deleted in HCC (46, 47), we investigated the LOH status of the HHIP locus. LOH analysis revealed that 31.3% (10 of 32) of the HCC tissues exhibited LOH at the HHIP locus, and the HHIP transcription level was significantly lower in HCC tissues showing LOH of HHIP than in HCC tissues showing retention of heterozygosity of HHIP. These results suggest that LOH is one of the mechanisms by which HHIP RNA is down-regulated in HCC.

For the 31 HCC tissues in which HHIP mRNA expression was down-regulated compared with the corresponding nontumor liver tissues, 7 tissues exhibited both HHIP DNA hypermethylation and LOH at the HHIP locus, 14 tissues showed only HHIP hypermethylation, and 3 tissues showed only LOH. To elucidate additional mechanisms for the down-regulation of the HHIP gene, mutational analysis was performed. Although this analysis was not performed with HCC tissues, we detected no mutation in the HHIP gene in pancreatic cancer cell lines and primary pancreatic cancers (32).

Although somatic inactivation of tumor suppressor genes is usually achieved by the loss of the chromosomal region that spans the first allele and by promoter hypermethylation or intragenic mutations in the second allele, some tumor suppressor genes may require only one genetic or epigenetic alteration if inactivation of one allele leads to haploinsufficiency of the protein (48). Therefore, HHIP may represent this type of tumor suppressor gene.

Seven HCC tissues in which HHIP mRNA expression was down-regulated, compared with corresponding nontumor liver tissues, showed neither HHIP DNA hypermethylation nor LOH. Although the cause of the HHIP down-regulation in these cases is unknown, the possible involvement of some novel somatic mutation and/or additional regulatory mechanisms, such as regulation by microRNA, might be worthy of consideration.

In previous studies, the overexpression of SMO or SHH was shown to be the major trigger for Hh signal activation in HCC (16, 17). However, the mechanisms of these overexpressions in HCC have not yet been determined. In addition, although the authors detected a novel mutation of SMO in a single HCC case (16), this type of mutation is rare and is less common than hypermethylation and/or LOH of HHIP.

In conclusion, we have shown that HHIP overexpression led to a reduction in hepatoma cell viabilities and that the restoration of HHIP transcription by demethylating agent in HHIP-hypermethylated cells attenuated Hh signaling. Moreover, we have shown that HHIP transcription is down-regulated and that down-regulation of HHIP transcription can be attributed to aberrant DNA hypermethylation or LOH of HHIP in a subset of hepatoma cell lines and in the majority of HCC tissues. Ectopic expression of SHH leads to ectopic HHIP expression, indicating that HHIP is a transcriptional target of Hh signaling (21). However, in the present study, we have shown that DNA hypermethylation and LOH are involved in the Hh signal-independent regulation of HHIP transcription in HCC. Hh signal activation through the inactivation of HHIP may have implication for the pathogenesis of human HCC.

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