

Table 1. Tumor-initiating ability of SP cells in the NOD/SCID xenograft transplant model

| | No. implanted cells | | | | |
|------------------|---------------------|---------------------|---------------------|---------------------|---------------------|
| | 100 | 1 × 10 ³ | 1 × 10 ⁴ | 1 × 10 ⁵ | 1 × 10 ⁶ |
| Huh7 | | | | | |
| <i>sh-Luc</i> | | | | | |
| SP cells | 0/5 | 14/15 | 13/13 | | |
| Non-SP cells | | 0/15 | 0/14 | 0/10 | 0/5 |
| <i>sh-BMI1-1</i> | | | | | |
| SP cells | 0/5 | 0/6 | 3/6* | | |
| Non-SP cells | | 0/6 | 0/6 | 0/5 | 0/5 |
| <i>sh-BMI1-2</i> | | | | | |
| SP cells | 0/5 | 0/9 | 0/8 | 1/3 [†] | |
| Non-SP cells | | 0/9 | 0/8 | 0/5 | 0/5 |
| PLC/PRF/5 | | | | | |
| <i>Sh-Luc</i> | | | | | |
| SP cells | 0/5 | 14/14 | 14/14 | | |
| Non-SP cells | | 0/14 | 0/14 | 0/10 | 0/5 |
| <i>sh-BMI1-1</i> | | | | | |
| SP cells | 0/5 | 0/5 | 2/5* | | |
| Non-SP cells | | 0/4 | 0/5 | 0/5 | 0/5 |
| <i>sh-BMI1-2</i> | | | | | |
| SP cells | 0/5 | 0/9 | 0/9 | 1/2 [†] | |
| Non-SP cells | | 0/9 | 0/9 | 0/5 | 0/5 |

NOTE: Tumor initiation was monitored for 14 wk after implantation.

*Delayed tumor formation and a decrease in tumor size were observed compared with tumors derived from control SP cells.

[†]Immunohistochemical data for tumors are displayed in Supplementary Fig. S3.

the impaired ability of Huh7 SP cells to self-renew. Thus, BMI1 might function in the maintenance of HCC cancer stem cells in both an *INK4A/ARF*-dependent and an *INK4A/ARF*-independent manner.

Finally, the present loss-of-function and gain-of-function assays revealed that BMI1 determines the self-renewal capability of SP cells, which directly contributes to the tumorigenic potential. However, further analysis will definitely be necessary to determine the role of BMI1 in primary HCC cancer stem cells (37). Our findings also indicate BMI1 to be a novel therapeutic target for the eradication of cancer stem cells in HCC. It would be of paramount importance to understand differential functions and targets of BMI1 in normal and cancer stem cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Down-Regulation of Hedgehog-Interacting Protein through Genetic and Epigenetic Alterations in Human Hepatocellular Carcinoma

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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.

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 smoothed (*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

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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., SHH, Indian Hh, and Desert Hh, with affinity similar to that of PTCH (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 GeneCopoeia and inserted it into the pCMV-Tag4 mammalian expression vector (Stratagene), thereby generating the pCMV-Tag4-HHIP vector. After confirming the insertion by sequencing, we transfected the pCMV-Tag4-HHIP vector or the empty vector (pCMV-Tag4) using Fugene 6 (Roche) into HuH7 and 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 800 µg Geneticin (Wako). Geneticin-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-HH1P1, H7-HH1P2 and 3B-HH1P1, 3B-HH1P2) 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).

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 $0.5 \mu\text{g}$ of $8 \times 3'$ GLI-BS-851 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 δ -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 AQueous 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 3.0×10^4 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 5.0×10^5 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).

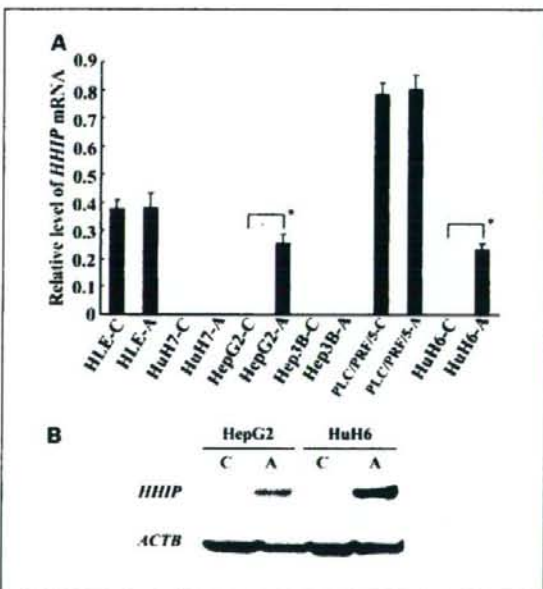


Fig. 1. A, relative levels of HHIP mRNA expression in hepatoma and hepatoblastoma cell lines. Columns, mean from the results of three independent experiments; bars, SD. *, $P < 0.0001$. B, HHIP protein expression in HepG2 and HuH6 cells with or without 5-aza-CdR treatment: C, control (without 5-aza-CdR treatment); A, treated with 5-aza-CdR.

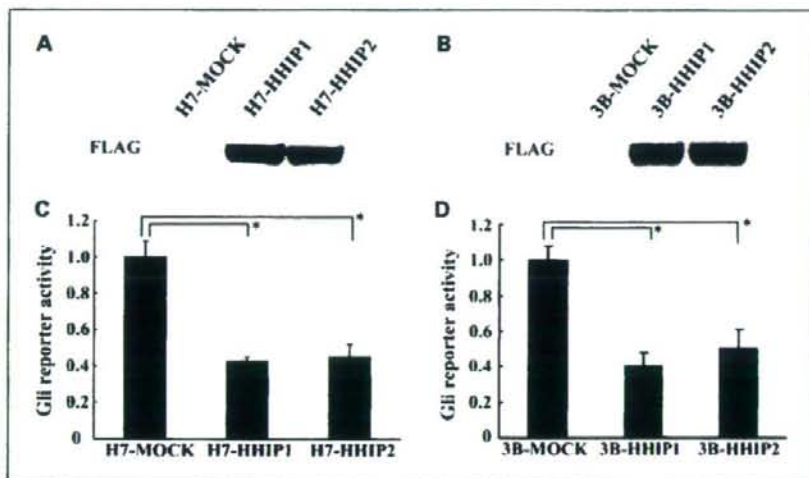


Fig. 2. A and B, establishment of *HHIP* stable transfectant was confirmed by Western blotting. C and D, Gli reporter activity of *HHIP* stably expressing cells. Ratios to the activity of MOCK cells. Columns, mean from three independent experiments; bars, SD. *, $P < 0.001$.

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*, Hs9999903_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'-AGTAGTYGGGTAGTTTGGAAAT-3'; second amplification, forward 5'-CGTTTATTTTGGAGGTTGGT-3'; and first and second amplifications, reverse 5'-CAACCAGAAATATTCATCTATCC-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

methylation 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 $\geq 4\%$, 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'-GTTGTAGTCGGTAGAGGAGATT-3', reverse 5'-ACAAATATTCATTAT CCGTATAAC-GAA-3', and probe 5'-FAM-AGTTTAGCGTTGGTTTTG-MGB-3'; for *ACTB*, forward 5'-TGGTGATGGAGGAGGTTAGTAAGT-3', reverse 5'-AACCAATAAACCTACTCTCCCTTAA-3', and probe 5'-FAM-ACCAC-CACCC AACACACAATAACA 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'-TCGTGCCCAAGT-3' and reverse 5'-TTGCTCACAGGATGCTTCT-3'; for D4S2998, forward 5'-AAGTCTTGGGCGCAG-3' and reverse 5'-TTCTACCCAGGG-GAACC-3'; for D4S1586, forward 5'-GCATGTACCATGCCCAGG-3' and reverse 5'-CCCAGAGTGCTGATGTGTG-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/PLF/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 cyclopamine treatment. To explore the relationship of *HHIP* expression and the sensitivities to cyclopamine in hepatoma and hepatoblastoma cell lines, we investigated the viabilities of cyclopamine-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 $\mu\text{mol/L}$ of cyclopamine ($P < 0.0001$), whereas treatment with 10 $\mu\text{mol/L}$ of cyclopamine 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.

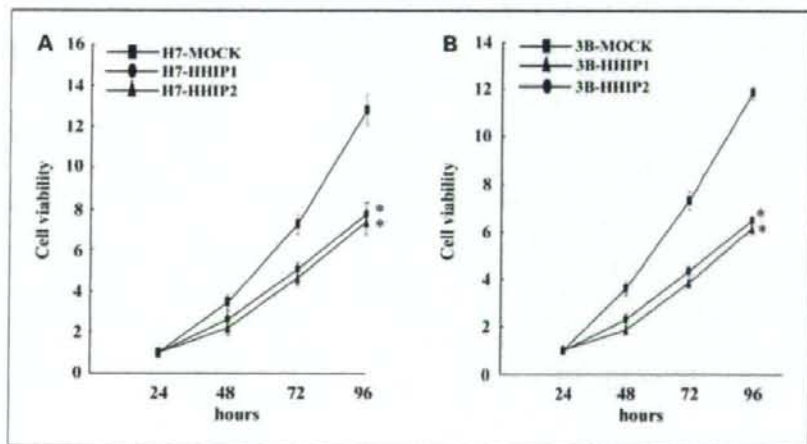


Fig. 3. Viabilities of *HHIP* stably expressing cells. A, HuH7 cells. B, Hep3B cells. Points, mean from three independent experiments; bars, SD. *, $P < 0.001$.

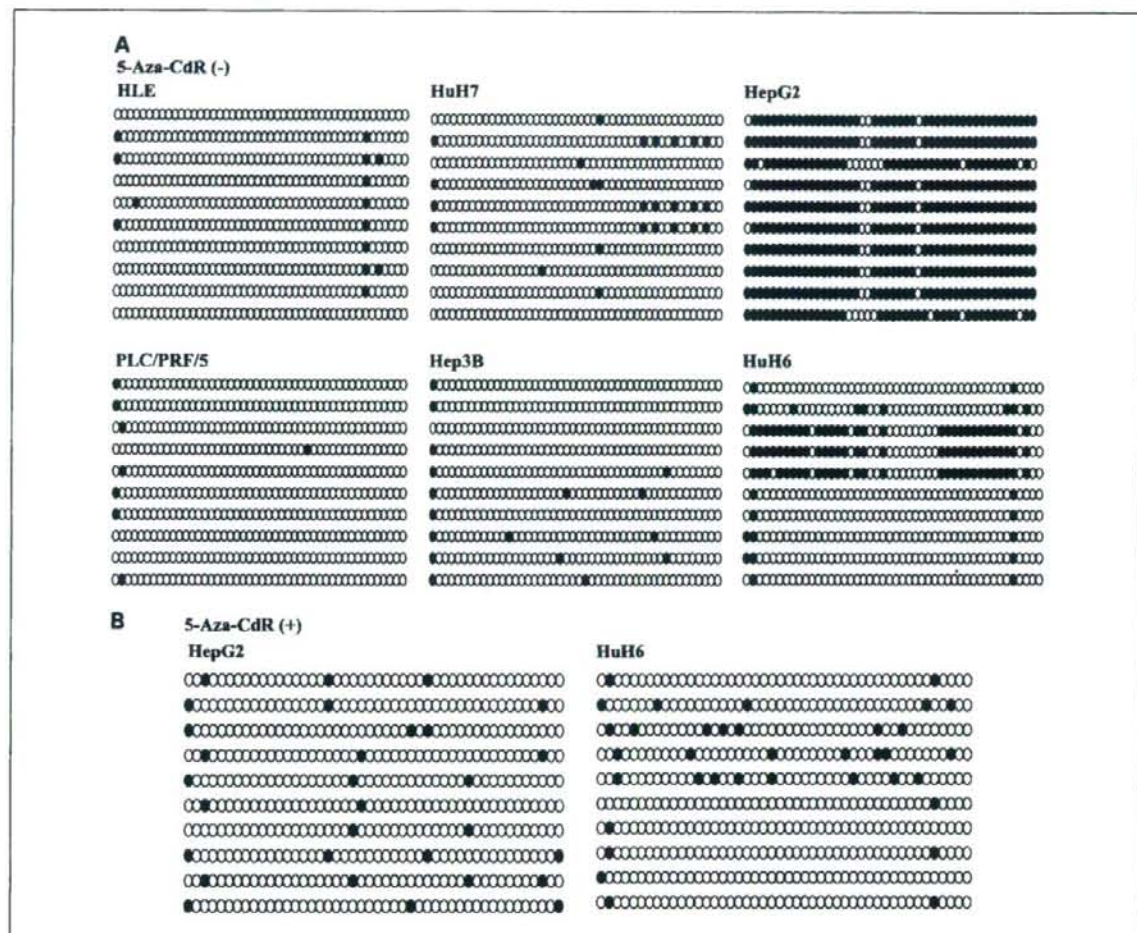


Fig. 4. Methylation status of 46 CpG dinucleotides in the promoter region of the *HHIP* gene. *A*, without 5-aza-CdR treatment; *B*, with 5-aza-CdR treatment. Each circle indicates a CpG site in the primary DNA sequence, and each line of circles represents the analysis of a single cloned allele. Closed circles, methylated CpG dinucleotides; open circles, unmethylated CpG dinucleotides.

***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

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

| Case no. | DNA methylation | | LOH | mRNA expression (T/NT) |
|----------|-----------------|----|-----|------------------------|
| | T | NT | | |
| 1 | + | - | + | 0.006 |
| 2 | + | - | + | 0.001 |
| 3 | + | - | - | 0.055 |
| 4 | - | - | - | 1.125 |
| 5 | + | - | + | 0.127 |
| 6 | - | - | - | 0.303 |
| 7 | + | - | - | 0.028 |
| 8 | - | - | - | 0.295 |
| 9 | + | - | - | 0.051 |
| 10 | + | - | NI | 0.008 |
| 11 | + | - | - | 0.044 |
| 12 | + | - | + | 0.016 |
| 13 | - | - | + | 0.033 |
| 14 | + | - | - | 0.002 |
| 15 | - | - | - | 1.079 |
| 16 | - | - | - | 1.093 |
| 17 | + | - | - | 0.004 |
| 18 | - | - | - | 2.396 |
| 19 | + | - | - | 0.007 |
| 20 | - | - | - | 0.050 |
| 21 | - | - | - | 0.209 |
| 22 | + | - | + | 0.001 |
| 23 | + | - | - | 0.004 |
| 24 | + | - | + | 0.0024 |
| 25 | + | - | NI | 0.009 |
| 26 | - | - | - | 0.707 |
| 27 | + | - | - | 0.012 |
| 28 | + | - | + | 0.012 |
| 29 | + | - | - | 0.007 |
| 30 | + | - | - | 0.002 |
| 31 | - | - | - | 3.889 |
| 32 | + | - | - | 0.009 |
| 33 | - | - | + | 0.104 |
| 34 | - | - | NI | 0.011 |
| 35 | - | - | NI | 0.095 |
| 36 | - | - | + | 0.084 |

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.

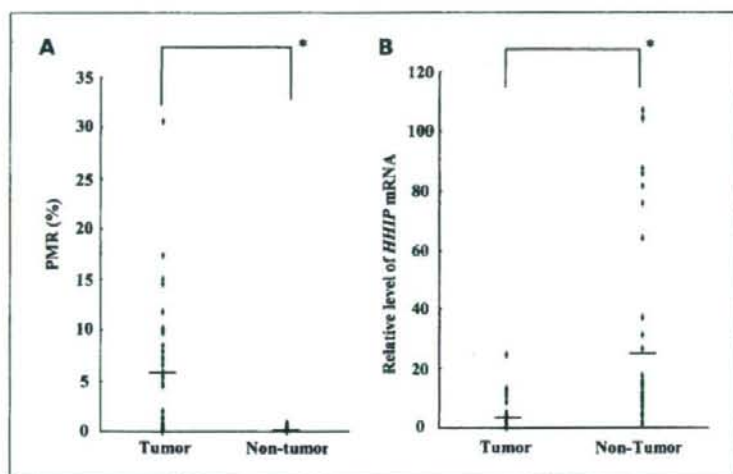


Fig. 5. A, methylation status of *HHIP* in 36 HCC tissues and corresponding nontumor liver tissues, as detected by quantitative real-time methylation-specific PCR (Methylight). PMR, percentage of fully methylated reference. Bar in the middle, mean percentage of fully methylated reference; points, mean from triplicate assays. *, $P < 0.0001$. B, *HHIP* mRNA expression in 36 HCC tissues and corresponding nontumor tissues. Bar in the middle, mean level of *HHIP* mRNA expression; points, mean from triplicate assays. *, $P < 0.0001$.

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 nontumor 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*, *p16^{INK4a}*, *SOCS*, *14-3-3 σ* , 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 nontumor 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

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.⁴ 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 gene 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 mRNA 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 any of the 13 exons of the HHIP gene in all the hepatoma and hepatoblastoma cell lines. Similarly, a recent study found 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.

Acknowledgments

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⁴ M. Tada, F. Kanai, Y. Tanaka, M. Senada, et al., unpublished data.

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Review Article

Current antiviral therapies for chronic hepatitis B

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Among current treatment options for chronic hepatitis B, nucleoside/nucleotide analog therapy has better tolerability and most patients respond to the therapy, while interferon (IFN) therapy has rather severe side-effects and a lower response rate. However, nucleoside/nucleotide analog therapies have problems of the emergence of drug resistance and poor sustainability of response after discontinuation. After the first nucleoside/nucleotide analog lamivudine, adefovir and entecavir are now utilized in many countries. Adefovir has efficacy for lamivudine resistant patients and current data suggests that adding adefovir to ongoing lamivudine is better than switching to adefovir in terms of viral suppres-

sion and the occurrence of resistance. Entecavir can be the first choice for naive patients, although cross-resistance has been known for lamivudine resistant patients and mutational screening should take place before using entecavir with such patients. Many other new nucleoside/nucleotide analogs are being developed such as telbivudine, clevudine and tenofovir; the details of each drug will be disclosed in near future.

Key words: Lamivudine, adefovir, entecavir, telbivudine, clevudine, tenofovir

INTRODUCTION

THE TREATMENT OF chronic hepatitis B remains a worldwide agenda. Two billion people are estimated to be infected with the virus and about 400 million of these have chronic hepatitis B.¹ Chronic hepatitis B is a common cause of mortality, responsible for 0.5-1.2 million deaths annually from liver cirrhosis and hepatocellular carcinoma (HCC).²⁻⁴ As hepatitis B virus (HBV) itself is not a cytotoxic virus, chronic hepatitis B is thought to be caused by an immunological response of the host against the viral proteins.⁵⁻⁷ Therefore the suppression of HBV replication is important for the treatment of chronic hepatitis B from the point of preventing the formation of viral proteins. Circulating HBV particles enter into the hepatocytes and after the formation of complete double-stranded DNA, covalently closed circular DNA (cccDNA) is made from the relaxed circular DNA.⁸ The production of pregenome RNA from the cccDNA is

important for active HBV replication.^{9,10} HBV has a reverse-transcription process from the pregenome RNA to HBV-DNA in its replication cycle,¹¹ and this process would be blocked by reverse transcriptase inhibitors. Current major treatment options are immunomodulatory and viral suppressant drugs, interferon (IFN)/pegylated (PEG) IFN or nucleoside/nucleotide analogs. These two types of therapies have different effects, each with their own merits and demerits. In this review, treatment with nucleoside/nucleotide analogs, especially drugs recently approved in Japan - adefovir and entecavir - are discussed.

AIM OF TREATMENT

THE MAJOR AIM of therapy for HBV is to prevent the progression of the disease to cirrhosis, end stage liver disease or HCC.¹²⁻¹⁵ The ideal end point of the treatment is HBsAg loss and the formation of anti-HBsAb, however, this rarely happens.¹⁶ Practically it is reasonable to infer improvement in disease outcome by suppressing HBV replication, with an accompanying improvement in serum alanine aminotransferase (ALT) and hepatic necroinflammation¹⁷ and, in HBeAg positive patients, followed by HBeAg loss or HBe seroconversion.

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TREATMENT OPTIONS

TWO MAJOR GROUPS of antiviral therapies are currently utilized for the treatment of HBV, IFN- α or PEG IFN- α , and nucleoside/nucleotide analogs. Treatment with IFN/PEG IFN- α is of finite duration and the response is often durable after treatment, however this treatment has side-effects and a relatively small number of patients respond, although higher viral clearance can be expected than with nucleoside/nucleotide analogs.¹⁶ Recent studies have revealed that the response to IFN is different among HBV genotypes. We can expect a better sustained response to standard IFN- α in HBeAg positive genotype B patients than genotype C patients, and in genotype A patients than genotype D patients.¹⁹⁻²¹ With PEG IFN- α , the HBe Ag seroconversion rate was 47%, 44%, 28% and 25% in genotype A, genotype B, genotype C and genotype D patients, respectively.²² The distribution of HBV genotypes differs in each area of the world.²³⁻²⁴ In Japan, 85% of chronic hepatitis B patients are genotype C, which is known to have higher risk of HCC at an earlier age than genotype B,²⁵⁻²⁷ and the effect of IFN is limited, so in such areas other therapies may be required.²⁸⁻²⁹

In contrast, nucleoside/nucleotide analogs are well tolerated and most patients respond to therapy, but treatment is hampered by the selection of drug resistant mutants, leading to a loss of efficacy and frequent relapse after discontinuation. Usually, cccDNA is resistant to the treatment and remains in the hepatocytes even after long-term treatment, so by the discontinuation of the drugs the HBV replication restarts from the remaining HBV-RNA produced from the cccDNA and relapse occurs promptly.³⁰ A high level of HBV-RNA may predict the early emergence of a resistant strain.³¹ So far the efficacy of nucleoside/nucleotide analogs has been reported to be almost the same among genotypes, but each genotype showed a difference in the occurrence rate of resistant mutants to lamivudine.¹² In addition, the durability of the therapy may be less in genotype C than in genotype B patients.³¹

Among nucleoside/nucleotide analogs, lamivudine was the first drug to be utilized, and because of its potency, safety profile and relatively low cost, it has been and still is widely applied globally as the first choice of therapy.^{34,35} However, the relatively high occurrence of resistance is recognized; 70% within 4 years.³⁶⁻⁴⁰ It has been reported that there are differences in the occurrence of resistant strains among HBV genotypes. Studies from Japan showed that genotype C, genotype B_j and genotype B_a had resistance to lamivu-

dine in 50%, 28% and 13% of patients, respectively, within 2 years, and genotype A, which is now increasing in number in Japan, has an even higher rate of resistance.^{41,42} Genotypes will be one of the important factors in choosing the therapy as we accumulate more data.

Another important problem for lamivudine is how and when the treatment should be discontinued. Combinations with lamivudine, with interferon, or HBV vaccination have been tried but their effects are still unknown.^{43,44} There are reports that reduction of the levels of HBV core related antigen may suggest the timing of the discontinuation.^{45,46}

For patients who have already developed resistance to it, substituting or adding other antiviral agents is necessary. Now, many other new nucleoside/nucleotide analogs have been developed, and several studies are ongoing to decide which of the available nucleoside/nucleotide analogs should be the first line therapy, although it remains difficult to pinpoint, for there is insufficient data to compare each of them; more data is needed on drug efficacy and genotypes. The data currently available will be shown below.

ADEFOVIR

ADEFOVIR (DIPVOXIL) IS an orally bioavailable prodrug of adefovir, a phosphonate acyclic nucleotide analog of adenosine monophosphate.⁴⁷ Adefovir acts by selectively inhibiting the reverse transcriptase-DNA polymerase of HBV by direct binding in competition with the endogenous substrate deoxyadenosine triphosphate (dATP).⁴⁸ Adefovir lacks a-3'hydroxyl group and, after incorporation into the nascent viral DNA, results in the premature termination of viral DNA synthesis. Unlike other nucleoside analogs such as lamivudine, adefovir is monophosphorylated and is not dependent on initial phosphorylation by viral nucleoside kinases to exert its antiviral effect.

Adefovir monotherapy

With adefovir 1-year monotherapy, the decline of viral load was 3.6-5.7 log₁₀ copies/mL in HBeAg positive patients and 3.9 in HBeAg negative patients. HBV-DNA negativity of less than the lower limit of detection (LLD), which was 200 copies/mL by polymerase chain reaction (PCR), after 1 year of therapy was 28-39%. ALT normalization was achieved in 48-81% of HBeAg positive patients and 72% of HBeAg negative patients. HBeAg loss was reported in 13-24%, HBe seroconversion in 8-18% and HBsAg loss in 0% of patients.⁴⁹⁻⁵² There is no apparent difference in the efficacy of adefovir

treatment among HBV genotypes.⁵³ Adefovir showed no resistance after 1 year, but the rate of resistance increased to 1-3%, 11%, 18% and 28% at years 2, 3, 4 and 5, respectively.^{24,54,55}

Prolonged therapy with adefovir in HBeAg positive patients resulted in viral load below the LLD, 10^3 copies/mL, in 28% at year 1, 45% and 56% of patients in years 2 and 3, respectively. ALT levels became normal in 48%, 71% and 81% after 1, 2 and 3 years of treatment, respectively. Rates of HBeAg loss increased to 42% and 52% and HBe seroconversion rates increased 29% to 42% at years 2 and 3.⁵⁶

In HBeAg negative patients, prolonged adefovir therapy for 2 years showed little additional decline in viral load but consolidated the response to adefovir, as 71-75% of the patients had a viral load below the LLD, 10^3 copies/mL, and ALT normalization in 73-79%. In the long-term, up to 5 years of continuous treatment resulted in a viral load below the LLD, 10^3 copies/mL, in 78-79% at year 3, 65-68% at year 4 and 67% after 5 years. ALT levels were normal in 69-78% at year 3, 70-75% at year 4 and 69% after 5 years.³⁰

Longer term results indicate that continued therapy with adefovir resulted in the suppression of HBV-DNA with lower rates of resistance than lamivudine, although adefovir monotherapy may not suffice for patients with high levels of viral replication and requiring rapid suppression of HBV-DNA.

Adefovir for lamivudine resistant patients

Adefovir has proven to be effective for lamivudine resistant mutants.^{15,57-60} The current interest surrounds adefovir monotherapy vs. combination therapy with lamivudine, for lamivudine resistant patients. Adefovir monotherapy is able to suppress viral load by 2.4-4.0 \log_{10} copies/mL, while adefovir and lamivudine combination therapy suppressed viral load by 2.4-6.5 \log_{10} copies/mL in 1 year.^{26,61} A randomized study found no difference in viral decline between adefovir monotherapy and combination therapy, although other studies have found that after long-term treatment, combination therapy showed a stronger viral decline (4.3 \log_{10} copies/mL) than adefovir monotherapy (3.4 \log_{10} copies/mL) and higher rates of HBV-DNA negativity by PCR, 81% in combination therapy versus 40% in adefovir monotherapy, in patients with baseline viral load $\geq 5 \log_{10}$ copies/mL.⁶²

Most of the clinical reports showed no difference in adefovir treatment outcome between lamivudine resistant patients and naïve patients,^{26,27,63} although adefovir was reported to have some degree of cross-resistance

with lamivudine *in vitro*.⁶⁴⁻⁶⁶ However, in lamivudine resistant patients, compared to combination therapy with lamivudine, adefovir monotherapy showed a higher rate of resistance.^{24,26,29,67}

As we can see from the above, recent reports of the comparative study of adefovir monotherapy versus adefovir and lamivudine combination therapy showed that adding adefovir to ongoing lamivudine therapy is preferable to switching to adefovir monotherapy in terms of the potent antiviral effect and a lower rate of resistance.^{24,26,29,35,40}

ENTECAVIR

ENTECAVIR IS A cyclopentyl guanosine analog and is known to inhibit all three activities of the HBV polymerase/reverse transcriptase: base priming, reverse transcription of the negative strand from the pre-genomic messenger RNA and synthesis of the positive strand of HBV-DNA. Entecavir has been shown to reduce the viral antigens and cccDNA levels in liver samples of HBV-infected woodchucks.³⁰

Entecavir for naïve patients

With 1 year of entecavir (0.5 mg) therapy for HBeAg positive patients, HBeAg loss was reported in 22%, HBe seroconversion in 21% and HBsAg loss in 2%, respectively, of patients.³¹ The decline of viral load was 6.9 \log_{10} copies/mL for HBeAg positive patients and 5.0 \log_{10} copies/mL for HBeAg negative patients.³² ALT normalization was achieved in 68% of HBeAg positive patients and 78% of HBeAg negative patients. HBV-DNA negativity, less than the LLD of 300 copies/mL by PCR, was achieved in 67% of HBeAg positive patients and 90% of HBeAg negative patients. Entecavir showed no resistance up to 2 years,³¹ but a cumulative resistance rate of approximately 1.2% after 4 years has been reported at the 42nd Meeting of the European Association for the Study of Liver Diseases.

Entecavir for lamivudine resistant patients

Entecavir shows efficacy for lamivudine resistant patients, although higher doses of entecavir (1.0 mg) are required and virologic rebound occurs due to resistance to entecavir. In a phase III trial, lamivudine resistant patients were treated with entecavir for 48 weeks. HBV-DNA was suppressed to less than the LLD, 300 copies/mL by PCR, in 19% of entecavir treated patients and 1% of lamivudine treated patients.⁴³ Another study

shows entecavir suppressed HBV-DNA less than the I.L.D., 300 copies/mL, in 21% and 34% of patients by weeks 48 and 96, respectively.⁷⁷

Entecavir is known to have cross-resistance with lamivudine and approximately 9% of lamivudine resistant patients treated with entecavir develop a resistance to entecavir after 2 years of therapy.⁴⁴ Virologic rebound due to entecavir resistance required pre-existing lamivudine resistance, HBV reverse transcriptase substitutions M204V and L180M, and additional changes at T184, S202, or M250.⁷³ A recent study showed T184, S202, or M250 substitution, which was considered to occur during entecavir therapy, emerged during lamivudine therapy before entecavir.⁴⁵

Entecavir is a potent inhibitor of HBV replication and for naïve patients entecavir showed a very low rate of resistance. Thus far entecavir can be considered the first line drug for naïve patients, although we may not put entecavir as the first line therapy for lamivudine resistant patients because of a relatively higher risk of cross-resistance and rebound. For lamivudine pretreated patients, screening of mutations may be necessary before switching from lamivudine to entecavir. Recent case reports suggest that entecavir resistant chronic hepatitis B can be successfully treated with adefovir and lamivudine combination therapy.⁷⁵

LIMITATIONS OF THE CURRENT THERAPIES AND NEWER NUCLEOSIDE/NUCLEOTIDE ANALOGS

Telbivudine

ANOTHER WELL-REPORTED nucleoside/nucleotide analog is telbivudine, a cytosine nucleoside analog. A dose-finding study revealed that this drug is a potent antiviral drug and dose-dependent antiviral activity is evident up to a dose of 400mg or more. In the 800 mg/d cohort, the mean reduction of HBV-DNA was 3.75 log₁₀ copies/ml at week 4.⁷⁶ A one-year trial of telbivudine 400mg/d or 600 mg/day, lamivudine 100mg/day, and the combination of these in HBeAg positive patients revealed the reduction of 6.43 or 6.09 log₁₀ copies/ml by telbivudine, respectively, compared to 4.66 of lamivudine, and 6.40 and 6.05 of combination at 52 weeks.⁷⁷ The randomized control trial of telbivudine or adefovir showed telbivudine demonstrated a greater reduction of HBV-DNA than adefovir at 24 weeks. After 52 weeks, the suppression was greater in patients who had received continuous telbivudine than in those who received continuous adefovir. This thus

showed telbivudine as a potent antiviral agent for HBV,⁷⁸ although it is associated with a rather high rate of resistance, 21.6% of HBeAg positive and 8.6% of HBeAg negative patients who received telbivudine for 2 years, and telbivudine-resistant mutations are cross-resistant with lamivudine.⁷⁹

Durability of response

Durability of response after discontinuation of therapy is one of the limitations of nucleoside/nucleotide analog therapy. In HBeAg positive patients who seroconverted during therapy, the response, which is assessed by HBeAg loss or HBe seroconversion, is durable in over half of the patients,^{42,80-83} but in HBeAg negative patients the durability of the response, which is the suppression of HBV-DNA, is quite poor. Patients treated with lamivudine for 2 years and who had undetectable HBV-DNA, less than 200 copies/mL, showed a 50% relapse rate 12 months after discontinuation.⁸⁴ With adefovir 96-week long-term therapy, 71% of HBeAg negative patients showed HBV-DNA levels that were less than 1000 copies/mL, but after discontinuation the majority of patients lost the benefit of treatment and only 8% of patients had levels below 1000 copies/mL at week 96 of follow up. Data on the durability of response to entecavir is lacking.

Clevudine is a pyrimidine analog and is reported to have relatively high sustained antiviral activity against HBV, or slow viral rebound after therapy. At the American Association for the Study of Liver Diseases (AASLD) in 2006, it was reported that 31% of HBeAg positive patients and 92% of HBeAg negative patients had undetectable HBV 12 weeks after the end of treatment. In HBeAg positive patients, median serum HBV-DNA reductions from baseline at week 24 were 5.10 log₁₀ and viral suppression in the clevudine group was sustained after therapy, with 3.73 log₁₀ reduction at week 34 and 2.02 log₁₀ reduction at week 48. At week 24, 59.0% of patients in the clevudine group had HBV-DNA less than the I.L.D., 300 copies/mL.^{85,86} In HBeAg negative patients, median changes of HBV-DNA were -4.25 log₁₀ copies/ml and 92% of the patients had undetectable HBV-DNA by PCR assay at 24 weeks of 30mg/day of clevudine treatment and viral suppression was 3.11 log₁₀ reduction 24 weeks after the end of treatment.⁸⁷ Although further studies are needed to confirm this, analysis of the mechanism of slow viral rebound in clevudine therapy could lead to the development of new drugs that provide better a sustainability of response.

Viral clearance: HBs seroconversion

With the currently available nucleoside/nucleotide analogs, HBsAg loss is rare - less than 2% - which is almost the same rate observed in the natural history of the disease, as compared to IFN therapy, which shows a 3-10% HBsAg loss within 1 year of therapy and 11-32% in sustained responders.^{5,88-93}

However, Tenofovir, another new nucleoside/nucleotide analog, was reported to have achieved a 14% HBsAg loss, although only in a small cohort of 35 patients.⁹⁴ Tenofovir, which has related molecules and a similar mechanism of action to adefovir, inhibits reverse transcription by competing with the substrate deoxyadenosine 5'-triphosphate. A phase III controlled trial comparing the efficacy of adefovir and tenofovir in HBeAg positive and negative patients is ongoing and data from the previous studies with lamivudine resistant patients showed that tenofovir had a faster and greater suppression of HBV-DNA than adefovir.⁹⁵

Tenofovir is expected to be an important next applicable drug in HBV infection and also HIV/HBV coinfection.^{96,97} More studies have to be carried out to investigate the effects of the drug on HBsAg loss and that mechanism is to be explored.

CONCLUSIONS

OUR KNOWLEDGE OF HBV and the therapeutic options has been broadened over the past decades, but much of the natural history of HBV remains unknown and none of the current registered therapies for chronic HBV can be said to be ideal in terms of tolerability and efficacy.

Nucleoside/nucleotide analog therapy has a high relapse rate after discontinuation and results in long-term therapy, which may put unborn children or the patients themselves at risk. Identifying the factors that contribute to the sustained response will help us to select patients who would be able to stop the therapy without relapse.

The occurrence of resistance to nucleoside/nucleotide analogs is currently unavoidable. Several more nucleoside/nucleotide analogs will be available in the future and switching from one drug to another will rescue patients who develop a resistance, but to pick the best drug for the patients, we should accumulate a large number of data concerning mutations, drug resistance and cross-resistance, and establish the data base to make it possible to select drugs for the patients based on mutational patterns or genotypes.⁹⁴

In HBV therapy, viral clearance is the ultimate aim of treatment, although current therapies do not suffice for that purpose. It will be important to establish treatment strategies based on both viral characteristics and host immune systems, which are necessary for HBV eradication.

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CLINICAL STUDIES

Prevalence of diabetes mellitus and insulin resistance in patients with chronic hepatitis C: comparison with hepatitis B virus-infected and hepatitis C virus-cleared patients

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Abstract

Background/Aims: Our aim was to evaluate the relationship between hepatitis C virus (HCV) infection and development of diabetes mellitus (DM) or insulin resistance (IR) in comparison with hepatitis B virus (HBV) infection and eradication of HCV infection by interferon treatment. **Methods:** This study consisted of 952 outpatients, including 544 HCV-infected (HCV+chronic), 286 HBV-infected (HBV+chronic) and 122 patients whose HCV was cleared by interferon treatment (HCV+cleared) (diabetes study). Among 849 without overt DM, IR was assessed in 423 patients, including 232 HCV-infected (HCV+chronic), 135 HBV-infected (HBV+chronic) and 56 HCV-eradicated patients (HCV+cleared) (IR substudy). **Results:** The prevalence of DM in the HBV+chronic, HCV+chronic and HCV+cleared groups was 6.3, 13.6 and 9.0%, respectively (HBV+chronic vs HCV+chronic, $P < 0.005$), in the diabetes study, and the prevalence of IR in the HCV+chronic group (54.3%) was also higher than that in the HBV+chronic (36.3%) ($P < 0.005$) and HCV+cleared groups (35.7%) ($P < 0.05$) in the IR substudy. However, HCV infection was not shown to be independently associated with DM development [odds ratio (OR) 1.669; $P = 0.0936$] and with IR (OR 1.531; $P = 0.2154$) by multivariate analysis in comparison with HBV infection as control. **Conclusions:** HCV-infected patients showed a higher prevalence of DM and IR than those with HBV infection. However, in Japan, other confounding factors appeared to be more important risk factors for the development of disturbance in glucose metabolism.

Approximately 1.5 million people in Japan and 170 million people worldwide have been infected with the hepatitis C virus (HCV), and chronic HCV infection causes chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) (1, 2). In most western countries, HCV infection is becoming a major cause of HCC and liver transplantation (3).

It is estimated that seven million people are affected by diabetes mellitus (DM) in Japan (4). Type 2 DM is a lifestyle-associated disease and is increasing worldwide, including in Japan (5). The risk factors associated with type 2 DM include family history, age, gender, obesity, smoking and physical activity (6). A close association between DM and insulin resistance (IR) is also reported (7).

The liver is crucial to carbohydrate metabolism and glucose homeostasis, and hepatic dysfunction causes glucose abnormalities leading to DM, which is pre-

valent in chronic liver disease, and especially in liver cirrhosis (8,9). Cirrhotic patients have glucose intolerance attributable to IR, which is caused by a post-receptor defect, decreased binding of insulin to target tissues and inadequate response of β -cells to secrete insulin appropriately to overcome the defect in insulin action (10). It has also been reported that DM increases the risk of developing chronic liver disease and HCC (11), and DM has been associated with non-alcoholic fatty liver disease including non-alcoholic steatohepatitis (12). Thus, there is a close association between DM and liver diseases.

Recently, there has been growing evidence to suggest an association between HCV infection and DM. A high prevalence of DM has been reported among patients chronically infected with HCV in comparison with controls or other liver diseases (13–18), in addition to a high prevalence of HCV infection among