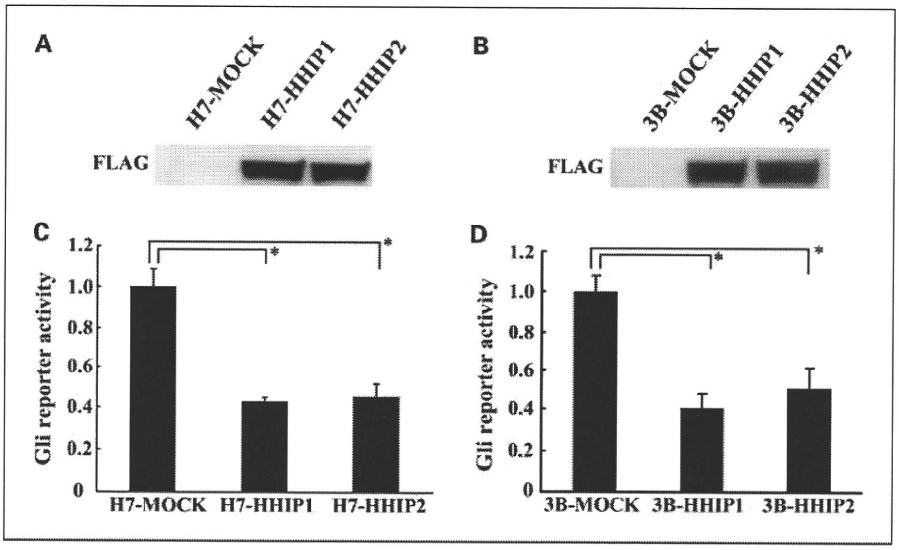

Fig. 2. *A* and *B*, establishment of *HHIP* stable transfectant was confirmed by Western blotting. *C* and *D*, Gli reporter activity of *HHIP* stably expressing cells. Ratios to the activity of MOCK cells. Columns, mean from three independent experiments; bars, SD. *, $P < 0.001$.

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*, 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′-AGTAGTYGGGTAGTTTYGGAATT-3′; second amplification, forward 5′-GGTTTAGTATTTTYGAGGTTGGT-3′; and first and second amplifications, reverse 5′-CAACCACAAATATTCACTTATCC-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′-GTTGTAGTCGTCGGTAGAGGAGATT-3′, reverse 5′-ACAAATATTCACTTAT CCGTATAACGAA-3′, and probe 5′-FAM-AGTTTAGCGTTGGTTTTG-MGB-3′; for *ACTB*, forward 5′-TGGTGATGGAGGAGGTTTAGTAAGT-3′, reverse 5′-AACCAATAAAACCTACTCCTCCCTTAA-3′, and probe 5′-FAM-ACCACCACCC 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′-TCGTGCCCAGCCAAGT-3′ and reverse 5′-TTGCTCACAGGATTGCTTCT-3′; for D4S2998, forward 5′-AAGTTCTTGGGCCGCAG-3′ and reverse 5′-TTCTACACCCAGGGGAACC-3′; for D4S1586, forward 5′-GCATGTACCATTGCCAGG-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 μmol/L of cyclopamine ($P < 0.0001$), whereas treatment with 10 μmol/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.

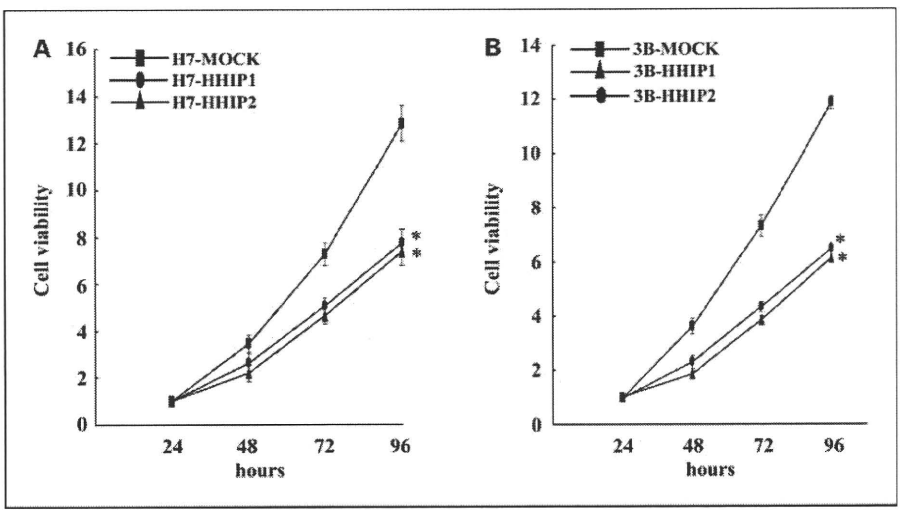

Fig. 3. Viabilities of *HHIP* stably expressing cells. *A*, HuH7 cells. *B*, Hep3B cells. Points, mean from three independent experiments; bars, SD. *, $P < 0.001$.

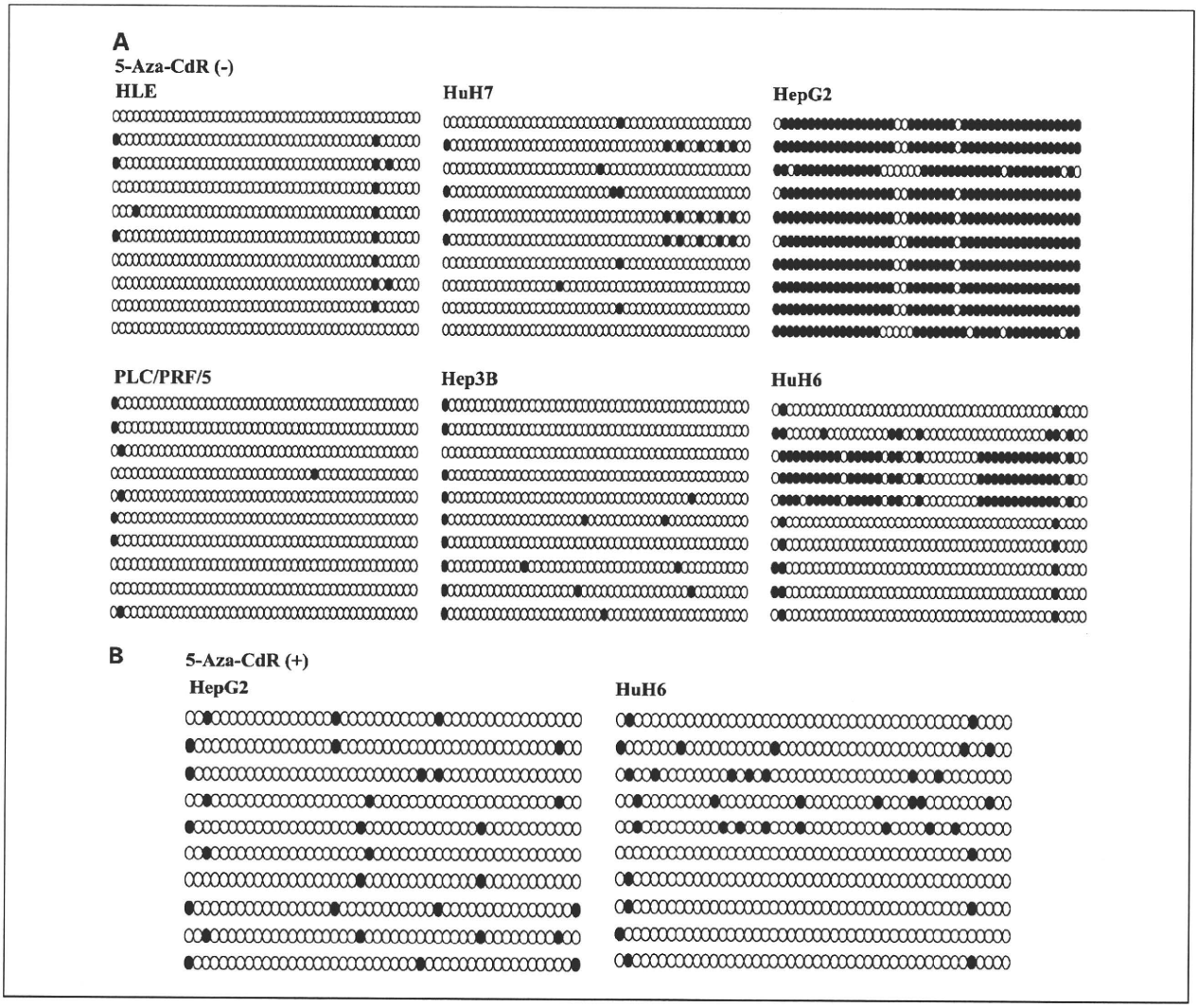

Fig. 4. Methylation status of 46 CpG dinucleotides in the promoter region of the *HHIP* gene. *A*, without 5-aza-CdR treatment; *B*, with 5-aza-CdR treatment. Each circle indicates a CpG site in the primary DNA sequence, and each line of circles represents the analysis of a single cloned allele. Closed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

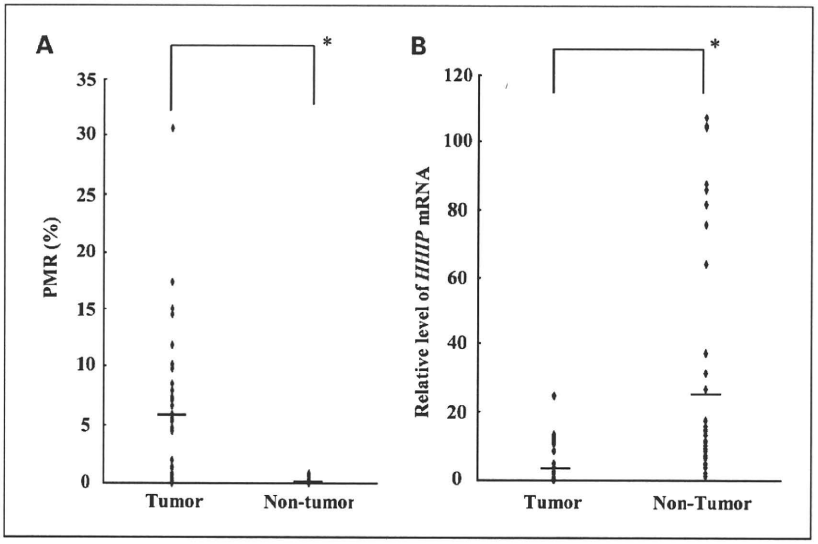

Fig. 5. *A*, methylation status of *HHIP* in 36 HCC tissues and corresponding nontumor liver tissues, as detected by quantitative real-time methylation-specific PCR (Methylight). PMR, percentage of fully methylated reference. Bar in the middle, mean percentage of fully methylated reference; points, mean 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 overexpression 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*, *14-3-3σ*, 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 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. Sanada, et al., unpublished data.

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Review Article

Current antiviral therapies for chronic hepatitis B

Mari Inada and Osamu Yokosuka

Department of Medicine and Clinical Oncology, Postgraduate School of Medicine, Chiba University, Chiba, Japan

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 suppression and the occurrence of resistance. Entecavir can be the first choice for naïve 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, telvibudine, clevudine, tenefovir

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.

Correspondence: Professor Osamu Yokosuka, Department of Medicine and Clinical Oncology, Postgraduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuou Ward, Chiba City, Chiba 260-8670, Japan. Email: yokosukao@faculty.chiba-u.jp
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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 necroinflammation[17] and, in HBeAg positive patients, followed by HBeAg loss or HBe seroconversion.

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.[18] 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.[32] In addition, the durability of the therapy may be less in genotype C than in genotype B patients.[33]

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 Bj and genotype Ba had resistance to lamivudine 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 DIPIVOXIL 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 $\log_{10}$ 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 ≥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 pregenomic 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,[33] 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 LLD, 300 copies/mL, in 21% and 34% of patients by weeks 48 and 96, respectively.[72]

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 $\log_{10}$ 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 $\log_{10}$ 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 $\log_{10}$ and viral suppression in the clevudine group was sustained after therapy, with 3.73 $\log_{10}$ reduction at week 34 and 2.02 $\log_{10}$ reduction at week 48. At week 24, 59.0% of patients in the clevudine group had HBV-DNA less than the LLD, 300 copies/mL.[85,86] In HBeAg negative patients, median changes of HBV-DNA were −4.25 $\log_{10}$ 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 $\log_{10}$ 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.[24]

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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TOPIC HIGHLIGHT

Harry HX Xia, PhD, MD, *Series Editor*

Current role of ultrasound for the management of hepatocellular carcinoma

Hitoshi Maruyama, Masaharu Yoshikawa, Osamu Yokosuka

Hitoshi Maruyama, Masaharu Yoshikawa, Osamu Yokosuka, Department of Medicine and Clinical Oncology, Chiba University Graduate School of Medicine 1-8-1, Inohana, Chuou-ku, Chiba 260-8670, Japan
Correspondence to: Hitoshi Maruyama, MD, Department of Medicine and Clinical Oncology, Chiba University Graduate School of Medicine 1-8-1, Inohana, Chuou-ku, Chiba 260-8670, Japan. maru-cib@umin.ac.jp
Telephone: +81-43-2262083 Fax: +81-43-2262088
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Abstract

Hepatocellular carcinoma (HCC) has a decisive influence on the prognosis of cirrhotic patients. Although α-fetoprotein (AFP) is a known and specific tumor maker for HCC, it is not suitable for the screening and surveillance of HCC because of its poor predictive value and low sensitivity. The use of imaging modalities is essential for the screening, diagnosis and treatment of HCC. Ultrasound (US) plays a major role among them, because it provides real-time and non-invasive observation by a simple and easy technique. In addition, US-guided needle puncture methods are frequently required for the diagnosis and/or treatment process of HCC. The development of digital technology has led to the detection of blood flow by color Doppler US, and the sensitivity for detecting tumor vascularity has shown remarkable improvement with the introduction of microbubble contrast agents. Moreover, near real-time 3-dimensional US images are now available. As for the treatment of HCC, high intensity focused ultrasound (HIFU) was developed as a novel technology that provides a transcutaneous ablation effect without needle puncture. These advancements in the US field have led to rapid progress in HCC management, and continuing advances are expected. This article reviews the current application of US for HCC in clinical practice.

Key words: Ultrasound; Contrast agent; Hepatocellular carcinoma; Liver; Surveillance; Treatments

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INTRODUCTION

Hepatocellular carcinoma (HCC) is increasing worldwide and is one of the most common carcinomas in the eastern part of Asia[1]. As the prognosis of cirrhotic patients depends on the occurrence and progression of HCC, management of this neoplasm is a major issue in clinical practice. The recent popularization of periodic surveillance and the development of diagnostic capabilities have resulted in the discovery of increasing numbers of patients with small HCC nodules[2,3]. Although tumor markers may be helpful for the diagnosis of HCC, imaging modalities are essential for finding and characterizing this neoplasm[4,5].

On the basis of the continuing development of digital technologies, ultrasound (US) has also shown significant improvements within the last decade[6]. As for grey-scale imaging, tissue harmonic imaging (THI) has improved both lateral resolution and contrast resolution by narrowing the width of the US beam, with the reduction of reverberation and side-lobe artifacts. Since the margin and structure of tumor nodules have become clear, with distinct delineation[7-10], THI has become popular as part of the routine work of grey-scale US examination.

Color Doppler imaging provides real-time evaluation of the hemodynamics in liver tumors, and power Doppler mode has contributed to a better detectability of blood flow[11-15]. However, limitations in the detection of slow flow and vessels located deeply from the skin surface have prevented the wider application of Doppler mode in the evaluation of tumor hemodynamics[16-18]. Furthermore, artifacts caused by respiratory or cardiac motion sometimes affect the precise evaluation of hemodynamic information.

With these backgrounds, US contrast agents have been expected to improve the detectability of blood flow in liver tumors, since the first report about a US contrast agent by Gramiak *et al*[19]. From the late 1980s to the 1990s, grey-scale contrast-enhanced US with carbon dioxide gained broad attention as an echo-enhancing technique, with

high sensitivity for detecting tumor vascularity and high performance for the characterization of liver tumors[20,21]. However, this method requires an arteriography procedure because carbon dioxide is easily soluble in blood. The development of microbubble contrast agents together with peripheral venous injection was expected for practical use. In the late 1990's, a galactose-based US contrast agent (SHU 508, Levovist) was made available by Schering, Germany[22,23]. It was a long-awaited material that could provide a stable enhancement effect in abdominal organs with a peripheral injection. Subsequently, many microbubble contrast agents have been produced or are currently under development. At present, the application of Doppler mode alone for detecting tumor blood flow is rare, as contrast-enhanced US with microbubble contrast agents provides details of the hemodynamics that are useful for the detection and characterization of liver tumors. Additionally, three-dimensional US images are now easily available due to the development of advanced digital technologies[24,25], and high intensity focused ultrasound (HIFU) was developed as a novel treatment method for tumors[26]. This article reviews the current development and application of US for the diagnosis and treatment of HCC.

SURVEILLANCE FOR HCC

Viral-related and/or alcoholic chronic liver disease is a high-risk factor for developing HCC that limits the prognosis. There is no question about the importance of periodic surveillance for HCC in these high-risk patients[27-29]. Some serum markers are known for HCC, and α-fetoprotein (AFP) is widely used for its diagnosis[30-32]. Ishii *et al* reported that sensitivity and specificity of AFP was 13.8% and 97.4% at a cut-off value of 200 ng/mL, respectively, and 62.1% and 78.3%, at a cut-off value of 20 ng/mL, respectively[31]. They added that when AFP and another tumor maker, protein induced by vitamin K absence or antagonist II (PIVKA-II), were combined with cut-off values of 40 ng/mL for AFP and 80 mAU/mL for PIVKA-II, sensitivity was 65.5% and specificity was 85.5%. The study by Tong *et al* showed that the positive predictive value for AFP to detect HCC was only 12% or less for all AFP cut-off values, and the maximum joint sensitivity and specificity as determined by receiver operator characteristic (ROC) analysis were approximately 65% and 90%, respectively. Meanwhile, the positive predictive value for US to detect HCC was 78%, while sensitivity and specificity were 100% and 98%, respectively[33]. They concluded that AFP should not be used as the only test for screening and surveillance for HCC because of its poor predictive value and low sensitivity. Larcos *et al* also mentioned that US screening was superior to AFP assay for detection of HCC[34]. Novel serum markers with improved sensitivity are awaited for screening tests for HCC.

US is the most common method for the screening of HCC because of its advantages - simple, non-invasive and real-time observation[4,5]. However, there has been a variety of results in the application of US for HCC surveillance (Table 1). Sherman *et al* reported that US showed a sensitivity of 71.4%, a specificity of 93.8%, with only 14% of positive predictive value, as a screening test in chronic HBsAg carriers[35]. Chalasani *et al* compared the sensitivity in a screening program between US and computed tomography (CT), and the sensitivity of US (59%) was much lower than that of CT (91%)[36]. Two other studies in the diagnosis of HCC before liver transplantation resulted in similar sensitivity between US and CT, 79.4% for US and 81.6% for CT[37], 58% for US and 53% for CT[38], respectively, with the latter claiming that US is preferable to CT for routine screening of HCC before liver transplantation because of its lower cost. Meanwhile, Teefey *et al* mentioned that the sensitivity of US (89%) was much higher than CT (67%) and magnetic resonance imaging (MRI, 56%)[39]. Evaluation of the actual sensitivity of US and other imaging techniques from the published studies on screening and surveillance is quite difficult because of the lack of a defined gold standard, as was also noted in the review article by Bolondi[28]. In addition, Chalasani *et al* described in their study that the lesser steatosis to change liver echogenecity in Asian patients with predominantly viral cirrhosis, leaner body habitus in Japanese patients resulting in better visualization of the liver by US, and differences in US technique between physicians (Japan) and technologists (USA) were the causes for the high detection rates by US in Japanese reports[4,36,40]. Although it is natural that US results depend on the physical size of the patients and the operator's skill, medical staffs and engineers who engage in US should not accept the current situation. Further technical and technological improvements are required to overcome these problems.

Tumor detectability between US without enhancement and contrast-enhanced spiral CT has been compared in some previous studies. The comparison may not be on an equal footing, as US has now acquired collaboration with microbubble contrast agents. The application of contrast-enhanced CT for screening of HCC would be expensive and invasive, and MRI has the limitation of a low availability rate of the equipment. Although contrast-enhanced US may not be cheap, it is much less invasive and more convenient than contrast-enhanced CT. The

Table 1 Sensitivity and specificity of US and other imaging modalities for the screening of HCC

Authors	US		Other modalities	
	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
Sherman *et al*[35]	71.4	93.8	-	-
Chalasani *et al*[36]	59	93	91 (CT)	96 (CT)
[1]Yao *et al*[37]	79.4	-	81.6 (CT)	-
			88.9 (MRI)	-
Gambarin-Gelwan *et al*[38]	58	94	53 (CT)	94 (CT)
[2]Teefey *et al*[39]	89	75	67 (CT)	75 (CT)
			56 (MRI)	81 (MRI)
			0 (PET)	88 (PET)

[1]Sensitivity of radiologic procedures in the diagnosis and staging of known HCC before liver transplantation. [2]The higher value was presented from two data obtained between two observers.

establishment of surveillance based on both non-contrast US and contrast-enhanced US may be necessary for the screening procedure of HCC.

According to clinical studies concerning the doubling time of tumor, median days were reported as 117 d (29-398 d) by Sheu *et al*[27] or 171.6 d (27.2-605.6 d) by Barbara *et al*[41], and the former study called for a suitable screening interval for the early detection of HCC of 4-5 mo. Solmi *et al* reported that the percentage of detected unifocal tumors with a diameter less than or equal to 3 cm was significantly higher in the group followed-up every six months by both US and AFP than the group without this follow-up protocol[42]. Depending on the risk factors, a score based on certain clinical findings may be predictive for the doubling time of HCC[41,43]. The latter report recommended a regular US follow-up of a 3- or 6-mo interval according to the risk of HCC development, sex (male), alkaline phosphatase, AFP, γ-glutamyltransferase and albumin[43]. The study by Izzo *et al* also supported the 6-month surveillance by AFP and US for patients with severe chronic active hepatitis or liver cirrhosis[44]. However, Fasani *et al* reported that screening with US every six months may be inadequate for early detection of liver cancer in patients with multiple risk factors because multinodular HCC was under detected by US[45]. A tailor-made surveillance interval may be required according to the risk of HCC development.

Bolondi *et al* examined their surveillance program based on US and AFP at six-month intervals in 313 cirrhotic patients, reporting that the cumulative survival of the 61 patients with liver tumors detected by the surveillance program was significantly longer than that of controls not participating in any specific surveillance program, with incidentally detected HCC, and multivariate analysis showed an association between surveillance and survival[46]. Other studies showed that surveillance based on US and AFP every 6-12 mo improved the survival of patients[47,48].

As described above, the method and appropriate interval of surveillance have been discussed from the aspect of growth speed of HCC, detected number and size of HCC, and the risk of developing HCC. Furthermore, the significance of surveillance is well-supported by the improved survival rate. US should play a main role in the screening procedure of HCC.

DIAGNOSIS OF HCC

Imaging diagnosis of HCC is based on the presentation of characteristic hypervascular appearances in nodules. The European Association for the Study of the Liver (EASL) has documented the diagnostic criteria for HCC in a report for the clinical management of HCC[49]. Nodules larger than 2 cm with an arterial hypervascular pattern by two imaging techniques or by one imaging technique associated with an AFP level higher than 400 ng/mL was considered to be HCC in cirrhotic patients without needing confirmation by a positive biopsy. Four imaging modalities, US, spiral CT, MRI, and angiography, were recommended for evaluation of the vascularity of hepatic nodules in that article.

The advantages of US imaging consist of the simple and non-invasive demonstration of blood flow by real-time observation. US is a unique method that can evaluate blood flow direction under physiological condition. In contrast to focal nodular hyperplasia (FNH) with a centrifugal blood flow appearance (Figure 1), HCC has a characteristic hypervascular appearance with centripetal blood flow, and a basket pattern is one of the typical findings of HCC by color Doppler imaging[50-52]. The clinical application of microbubble contrast agents has resulted in remarkable improvement in blood flow detection by US examination. It was reported that the same enhancement pattern was found between contrast-enhanced harmonic grey-scale imaging with Levovist and contrast-enhanced helical CT in 53 of 61 (87%) HCC nodules[53]. Other studies have also shown over 80% concordance of tumor vascularity[54,55] between contrast-enhanced US with SonoVue (Bracco Diagnostics, Princeton, NJ, USA) and contrast-enhanced helical CT. Thus, the application of Doppler mode alone for detecting tumor blood flow is rare, as the more recent availability of microbubble contrast agents has assisted in overcoming the limitations of Doppler methods.

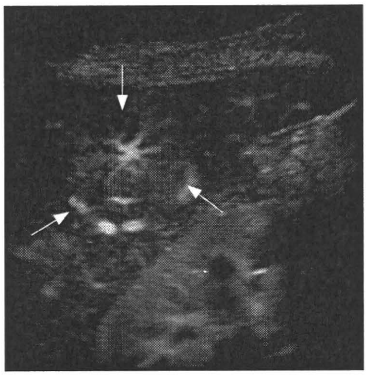

Figure 1 Contrast-enhanced harmonic imaging with Sonazoid in focal nodular hyperplasia (FNH). The centrifugal blood flow appearance like "spoke-wheel sign" was clearly demonstrated in the center of the nodules (arrows).

The diagnostic performance of contrast-enhanced US is not limited to the demonstration of tumor vascularity. Some microbubble contrast agents have a characteristic property of organ-specific accumulation[56-59]. Although the precise mechanism remains unclear, the reticuloendothelial system (i.e., phagocytosis by Kupffer cells) may be involved in this phenomenon. Both Levovist and Sonazoid (Nycomed-Amersham, Oslo, Norway) accumulate in the liver, and sonograms in this phase (late liver-specific parenchymal phase) are frequently used for the detection or characterization of liver tumors. In contrast, Definity (Bristol-Myers Squibb, N. Billerica, MA, USA) and SonoVue do not accumulate in the liver. The characterization of liver tumors by contrast-enhanced US has been carried out using accumulation images as well as vascular enhancement images (Figure 2A and B).

Concerning the discrimination of malignant versus benign liver lesions by contrast-enhanced US, recent literature has reported sensitivity of 98% to 100% and specificity of 63% to 93% with Levovist[60-63], and sensitivity of 98% and accuracy of 92.7% with SonoVue[64]. Furthermore, in a clinical study with two independent image reviewers, Kim *et al*[65] described that contrast-enhanced US (agent detecting imaging mode with Levovist) provided a specific diagnosis in 75%-79% of 75 patients with focal hepatic lesions, and that the technique

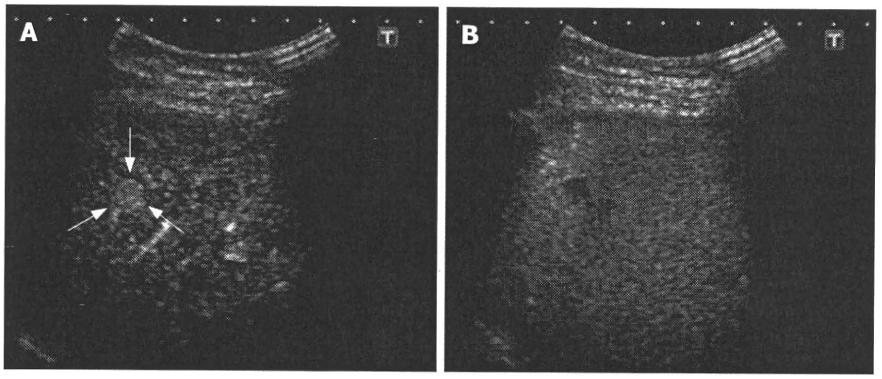

Figure 2 Contrast-enhanced harmonic imaging with Sonazoid in small HCC (9.8 mm, arrows). A: Early-phase image (22 s after the injection); B: Late-phase image (10 min after the injection). The early-phase image showed positive enhancement and the late-phase image showed negative enhancement in the nodule. These findings could easily diagnose this lesion as HCC.

was successful as a confirmatory imaging technique in 63%-72% of the patients.

Hypervascular hepatic lesions do not always reflect the fact that the final diagnosis of the nodule is HCC in heavy drinkers[66], since benign hypervascular nodules sometimes occur in their liver. A recent report has shown that the ring-shaped appearance on liver-specific contrast-enhanced sonograms with Levovist may be a useful sign for the differential diagnosis of benign nodule from HCC in heavy drinkers[67]. Since contrast-enhanced CT hardly differentiates these benign nodules from HCC, this characteristic finding may prevent unnecessary treatments under misdiagnosis. Moreover, it could be expected to lead to a reduction in the application of percutaneous needle biopsy, an invasive procedure, for the precise diagnosis.

Non-hypervascular and/or small (< 2 cm) nodules

Well-differentiated HCC, dysplastic nodule (DN) and regenerative nodule (RN) do not always reveal the specific hypervascular pattern on contrast-enhanced CT such as typical HCC[68-71]. The characterization of such non-hypervascular nodules is very important in clinical practice[72,73] because high-grade DN are considered potentially pre-malignant lesions. However, as these non-hypervascular nodules have Kupffer cell distribution[74,75], observation of the superparamagnetic iron oxide-enhanced (SPIO) MR images or liver-specific images on contrast-enhanced US could not easily characterize them.

According to the EASL report, percutaneous needle biopsy has until now been a standard method for the diagnosis of non-hypervascular hepatic nodules or small hepatic nodules of 1 cm to 2 cm[49], because characterization of these nodules by imaging modalities alone is difficult[76-79]. As for nodules smaller than 1 cm, EASL recommended repeated US observation every 3 mo until the lesion grows to 1 cm, at which point additional diagnostic techniques can be applied[49].

Thanks to the establishment of US-guided needle puncture technique[80], percutaneous needle biopsy has a quite high diagnostic accuracy. Caturelli *et al* found that the typing accuracy of fine-needle aspiration biopsy was 88.6% for nodules with diameters < 10 mm, 86.2% for nodules with diameters of 11-15 mm, and 91.3% for nodules with diameters of 16-20 mm[81]. Durand *et al* reported that US-guided FNB diagnosed HCC nodules with a sensitivity of 91%[82]. However, liver biopsy for small nodules always has the possibility of sampling error, and a negative biopsy of a nodule visible with imaging techniques in a cirrhotic liver can never be taken as a criterion to rule out malignancy[83]. Additionally, as rapid progression is rare in these kinds of nodules, repeated observations in their clinical course would determine their management. Therefore contrast-enhanced US can be expected to be an effective diagnostic tool for these non-hypervascular lesions because of its high resolution and non-invasive procedure.

TREATMENT SUPPORT AND EVALUATION OF THERAPEUTIC EFFECT

US-guided treatment

Since the majority of HCC patients have poor liver function and recurrence is not rare, surgical treatment is not always an appropriate choice[2,3,49]. With such backgrounds, percutaneous ethanol injection (PEI)[84-86] and radio-frequency ablation (RFA)[87,88] were developed and came to be widely used in clinical practice as minimally invasive methods[89]. They are now a first-line, favored approach with an efficient therapeutic effect on HCC[90-93].

Treatment for recurrent lesions

Although percutaneous US-guided treatments provide sufficient therapeutic effect, recurrence often plagues many HCC patients. According to long-term study results, cumulative recurrence rates of the treated site of post-PEI lesions were 3.4% at 1 year, 7.1% at 2 years, and 10% at 3 years, and those of the untreated sites in liver were 18.7% at 1 year, 62.1% at 3 years, and 81.7% at 5 years, respectively[94]. Thus, many HCC patients have to receive repeated treatments during their clinical course. In order to minimize adverse effects to the liver, less invasive treatment such as PEI or RFA is preferable for these patients. However, localization of lesions on sonograms is sometimes problematic in patients with cirrhotic liver and/or repeated treatment history[95,96]. Although percutaneous treatment under CT guidance is a well-established technique and a useful method for lesions undetected by US, the method lacks convenience and exposes both patients and physicians to radiation[97-100]. Microbubble contrast agents are also useful in such a case. A recent study showed that contrast-enhanced US with Levovist could localize 24/32 (75%) of HCC lesions that were invisible by non-contrast US[101]. Application of the next-

generation US contrast agents, SonoVue and Sonazoid, is expected to improve the localization result.

Evaluation of therapeutic effect

US examination is eligible for the evaluation of the therapeutic effect after percutaneous treatments such as PEI and RFA, because they are usually performed under US guidance. In fact, contrast-enhanced US has come to be frequently applied for evaluation of the therapeutic response in HCC nodules with improved sensitivity and specificity for detecting tumor blood flow (Table 2). According to the results by Bartolozzi *et al*, color Doppler US with Levovist showed sensitivity of 92%, specificity of 100%, and accuracy of 98% compared to the results of spiral CT and biopsy, in the detection of residual tumor tissue in 47 HCC lesions after PEI[102]. Wen *et al* examined the efficacy of coded harmonic angio mode with Levovist for detecting residual tumor in 91 HCC nodules about one week after RFA in comparison with contrast-enhanced CT, and they found that sensitivity, specificity, and diagnostic accuracy of US were 95.3%, 100%, and 98.1%, respectively[103]. Meloni *et al* reported that sensitivity and specificity of pulse inversion harmonic imaging with Levovist were 83.3% and 100%, respectively, for detecting residual non-ablated tumor at 4 mo after treatment in 35 patients with 43 HCC nodules, compared with helical CT findings[104]. Immediate evaluation of the therapeutic effect is often desirable after RFA for the management of HCC, and Choi *et al* mentioned that diagnostic agreement between power Doppler with Levovist about half or one day after ablation therapy and CT just after ablation was achieved in 100% of the 45 HCC nodules in 40 patients[105]. Another study showed that diagnostic concordance between agent detection imaging with Levovist performed within 24 h after RFA and 1-mo follow-up CT was 99% in 90 patients with 97 HCC nodules[106]. Thus, estimation of the therapeutic response in HCC after percutaneous treatments would become more efficient on the basis of this non-invasive imaging method. Although artificial signals caused by the RFA procedure affect an early detailed observation[105-107], monitoring by contrast-enhanced US during RFA would likely be applied to the assessment of the therapeutic effect as well as the detection of viable tumor.

It is well known that contrast-enhanced CT can hardly evaluate intratumoral contrast enhancement when partial retention of iodized oil is present in the tumor after transcatheter arterial chemoembolization (TACE). Therefore, the therapeutic effect of TACE is usually assessed by the distribution of iodized oil in the tumor on non-contrast CT images, though these findings are an indirect presentation. As MRI findings are not affected by the presence of iodized oil, contrast-enhanced MRI is favorable for the assessment of the therapeutic effect after TACE. However, the equipment has not yet come into wide-spread use, the procedure is not convenient, and evaluation of the findings in small lesions is sometimes difficult due to the low resolution and influence of motion artifacts. Contrast-enhanced US has the advantage of not being limited by iodized oil deposition that affects the evaluation of contrast-enhanced CT findings. Some clinical studies have shown the magnitude of contrast-enhanced US for evaluation of the therapeutic effect after TACE[108,109]. According to the report by Pompili *et al*, contrast-enhanced US with SonoVue resulted in diagnostic agreement in 53/56 cases (94.6%), with 87.0% sensitivity and 98.4% specificity compared with contrast-enhanced CT findings, after non-surgical treatments for HCC[110]. Another study showed that contrast-enhanced US resulted in considerably higher sensitivity in detecting residual tumor blood flow after TACE than dynamic CT or dynamic MRI[111]. Meanwhile, Lim *et al* described that a reliable assessment of intratumoral blood flow by contrast-enhanced US may not be possible in many instances, particularly in small lesions or in lesions located deep within the liver parenchyma[112]. They concluded that CT is the standard imaging technique for monitoring the effectiveness of TACE and RFA, and contrast-enhanced US and MRI can complement CT in evaluating the therapeutic response. Although the performance of the US examination may depend on the operator's skill, location of the tumor and system capability, quite a few radiologists and hepatologists may believe that contrast-enhanced US plays a major role in evaluation of the therapeutic effect after TACE. The recent developments in this technology would allow contrast-enhanced US to be positioned as the standard method for evaluation of the therapeutic effect in many HCC patients (Figure 3A and B).

Table 2 Assessment of therapeutic response after percutaneous treatment for HCC using contrast-enhanced US

Author	Treatment	No. of patients/ No. of lesions	Results[1] (contrast agent)
Bartolozzi *et al*[102]	PEI	40/47	Sensitivity 92% Specificity 100% Accuracy 98% (Levovist)
Wen *et al*[103]	RFA	67/91	Sensitivity 95.30% Specificity 100% Accuracy 98.10% (Levovist)
Meloni *et al*[104]	RFA	25/43	Sensitivity 83.30% Specificity 100% (Levovist)
Choi *et al*[105]	RFA	40/45	Diagnostic agreement 100% (Levovist)
Kim *et al*[106]	RFA	90/94	Diagnostic concordace[2] 99% (Levovist)
Solbiati *et al*[107]	RFA	20/20[3]	Sensitivity 50% Specificity 100% Diagnostic agreement 85% (Levovist)
Pompili *et al*[110]	PEI, RFA, TACE Combined treatments	47/56	Sensitivity 87% Specificity 98.40% Diagnostic agreement 94.60% (SonoVue)

[1]Comparison with contrast-enhanced helical CT; [2]1-mo follow-up CT; [3]Solitary colorectal liver metastases.