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Methylation Inactivates Expression of CDP-diacylglycerol Synthase 1 (*CDS1*) in Hepatocellular Carcinoma

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Abstract. *Background: CDS1 is an enzyme required for the regeneration of the signaling molecule phosphatidylinositol-4,5-bisphosphate (PIP2) from phosphatidic acid. These phosphoinositides and their cleavage products are a class of second messengers, which are involved in cell growth and oncogenesis. The role of CDS1 in the development of hepatocellular carcinoma (HCC) was explored. Materials and Methods: The expression of CDS1 in 52 HCC and paired non-cancer tissues was examined by real-time quantitative reverse transcription-polymerase chain reaction analysis. Results: The results showed that the expression levels of CDS1 significantly decreased in HCC. However, no mutation was found within the coding region. Interestingly, in the promoter area of the CDS1 gene, most of the CpG sites were methylated in 73% of the cancer tissues; in contrast, only a partial methylation of CpG was found in 50% of the non-cancer tissues. Conclusion: Our results suggested that the down-regulated CDS1 expression in HCC was due to the inactivation of the CDS1 gene by methylation and that the differential expression correlated to the ratio of CpG sites being methylated.*

Phosphoinositide-mediated signaling pathways are a ubiquitous mode of intracellular signal transduction in eukaryotic cells. Phosphoinositides and their cleavage products are a class of second messengers that can be found downstream of many tyrosine kinase receptors. These second messengers are involved in cell growth, differentiation, development and oncogenesis (1-5). The signals from many of these different cascades converge when phospholipase C (PLC) is activated. PLC catalyzes the hydrolysis of the minor membrane phospholipid PIP2 into the second messengers inositol trisphosphate (IP3) and diacylglycerol (DAG). IP3 mobilizes internal stores of calcium, which affects and modulates many cellular processes; DAG activates members of the protein kinase C (PKC) family of proteins. Failure to regulate these second messengers can lead to severe cellular dysfunction. For example, hyperactivation of PKC may cause uncontrolled cell growth and tumorigenesis. PIP2 plays a central role in these signaling pathways, which are tightly modulated in the cell (6, 7).

CDP-diacylglycerol synthase (CDS) is important in controlling the activity of the phosphoinositide cycle in signal transduction systems (8-10). Altered phosphoinositide metabolism has been reported in carcinogen-induced hepatic tumors (11, 12), prostatic cancer cells (13) and mammary tumors (14). We suggest that disturbance of *CDS1* expression may play a role in the oncogenesis of some cancers. In order to prove this hypothesis, the expression status of *CDS1* in hepatocellular carcinoma (HCC) was analyzed and the mechanism of its *de novo* expression was explored.

Materials and Methods

Sample. Fifty-two resected primary HCC and nearby non-cancer tissue samples, both confirmed by histology, were obtained from 52 patients (42 men and 10 women) at Changhua Christian Hospital, Taiwan, ROC. The age of the patients ranged from 26 to 78 years, with a mean of 58 years. Clinically, three cases were Grade I, 20 cases were Grade II and 29 cases were Grade III. All the tumors were HCC. The proportion of tumor cells was near 100% in the tumor tissues. The tissues were frozen immediately after surgical resection and stored in liquid nitrogen until extraction of DNA or RNA. Three HCC cell lines (Huh-7, HepG2 and Mal-5) were also studied. The DNA extraction was performed as previously described (15). Total RNA was extracted by a commercial kit (RNA-Bee™, Tel-Test, Inc., TX, USA), and

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Key Words: CDP-diacylglycerol synthase 1, hepatocellular carcinoma, methylation, mutation analysis.

Table I. *The primers used for sequencing coding area of CDS1 gene.*

	Upstream primer	Downstream primer	Tm (˚C)
Exon 1	5'-TGCTTGCAGCTCAGGTTTCG-3'	5'-ACCCTTGCTCTCAAGTGTCC-3'	62
Exon 2	5'-TGAGATTTGACACACTGAGC-3'	5'-ACAAGGATAACCTCAAAGCC-3'	58
Exon 3	5'-GGCAAATCAATACTGAAACC-3'	5'-TGCTCAAATTACTTCAATCC-3'	58
Exon 4	5'-CTGCTTTATTGAATGATTAAGC-3'	5'-ATACTGACTGATCAAGACGC-3'	58
Exon 5	5'-AGTTTGAGGGGAAGGAAAGC-3'	5'-ATGAAAAAGAGAGGCTGGAAT-3'	58
Exon 6	5'-TGGTTTATTTGGGCTCAAGG-3'	5'-ACAGCTAGCAGATTCGACTG-3'	58
Exon 7	5'-GCTGTTGCTTCTTCAGCTGC-3'	5'-CTGTAAGACACTATCCAAGC-3'	56
Exon 8	5'-AACCTTTCCGAATATCTGCC-3'	5'-TGGAAATGTTCTCCAGTGGC-3'	60
Exon 9	5'-GAGATGTACAAGTTATTGAGG-3'	5'-AGACCAGTAAGCTTGGTATC-3'	62
Exon 10	5'-ATTCTATCTCTGGCCTTTCTG-3'	5'-ACAGAGGGAAACTCTGTCTC-3'	60
Exon 11	5'-TCTTAGATCAGGTGGTATGC-3'	5'-TGAGCGGACCAAATTCCAAC-3'	62
Exon 12	5'-ATGACGCCAAAGAGACACAG-3'	5'-ATTGTAGATTACCATTGACGG-3'	60
Exon 13	5'-ATTGGAGGTATGTGCTTCAC-3'	5'-CCAAAACTTCAACCTATTGC-3'	60

Tm=melting temperature.

stored as a pellet in ethanol or solubilized in RNase-free water and kept at –70˚C. This study was approved by the Institutional Review Board of Changhua Christian Hospital.

Real-time quantitative RT-PCR analysis. The mRNA sequence of the *CDS1* gene was evaluated and the specific forward and reverse primers and TaqMan probe were designed by Primer Express software (Applied Biosystems, Foster City, CA, USA). The probe was synthesized and labelled with appropriate fluorescent dyes (Applied Biosystems). The sequences of the forward and reverse primers and probe were as follows: CDS-1F: 5'-TCT TGA GAC AGG AAA GAG TGA GCT T-3', CDS-1R: 5'-TGG GCC ATT AAA GAT GCA AA-3', probe: 5'-FAM-CCC TTT CCA GAT CCA CAG CAT TGC A-TAMRA-3'.

For internal control of the RNA, the expression of glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*) was also examined by RT-PCR. The amount of *CDS1* was normalized to the endogenous reference *GAPDH* to obtain the relative threshold cycle (ΔC_t) and then related to the ΔC_t of non-cancer tissue to obtain the relative expression level ($2^{-\Delta\Delta C_t}$) of *CDS1*.

All reactions were performed in an ABI-7700 sequence detector (Applied Biosystems) and by TaqMan EZ RT-PCR core kit (Applied Biosystems), according to the manufacturer's protocol. RT-PCR was performed in a 25-μL final volume containing 400 nM of each primer, 200 nM probe, 300 μM of each deoxynucleoside triphosphate (dNTP), 3.0 mM manganese acetate, 2.5 U rTth DNA polymerase, and 1X PCR buffer. The RT-PCR cycling parameters were set as follows: the RT reaction at 50˚C for 2 minutes, 60˚C for 30 minutes and 95˚C for 5 minutes, followed by 40 cycles of PCR reaction at 94˚C for 20 seconds and 62˚C for 1 minute.

To determine the precision of the assay, four replicates of each sample of total RNA were run on four separate days. Intra-assay (within-run) precision was determined by calculating the mean, standard deviation (SD) and coefficient of variance (CV) of the threshold cycle (Ct) values for each sample and for each set of primers and probe on each day.

CDS1 gene mutational analysis. Amplification of the coding region of the *CDS1* gene was carried out by PCR, and the primers are shown in Table I. PCR was performed in a 50-mL final volume containing 200 nM of each primer, 200 mM of each dNTP, 3.5 mM $MgCl_2$, 2 U Taq DNA polymerase (Promega, Madison, WI, USA) and 1X PCR buffer. The amplification procedure was carried out as follows: 35 cycles of PCR reactions including denaturing at 95˚C for 1 minute, annealing at a temperature depending on the melting temperature (Tm) of each primer set for 1 minute, and extension at 72˚C for 2 minutes. The PCR products were subject to gel purification and direct sequencing. DNA sequencing was performed using the ABI Prism 310 Genetic Analyzer and the Big Dye Terminator cycle sequencing kit (Applied Biosystems), according to the manufacturer's protocol.

Methylation-specific PCR. Genomic DNA was modified with sodium bisulfite and methylation-specific PCRs were performed, as described by Herman *et al.* (16) with some modifications. The primers which detected the methylated CpG sequences were 5'-CGT TGT AGG CGG TTT CGA GC-3' (nt –215 to –196) and 5'-GCT CCC GCG CTA CCG TAA CG-3' (nt –6 to –25). The primers which detected the unmethylated sequences were 5'-TGT TGT AGG TGG TTT TGA GT-3' (nt –215 to –196) and 5'-ACT CCC ACA CTA CCA TAA CA-3' (nt –6 to –25). Briefly, approximately 4 μg of genomic DNA in 40 μL H_2O was denatured by incubation with 10 μL of 1 M NaOH at 37˚C for 10 minutes, and then modified with 30 μL of 10 mM hydroquinone and 520 μL 1.5 M sodium bisulfite (pH 5.0) at 50˚C for 16 hours. The DNA samples were eluted with 100 μL pre-warmed H_2O (65-70˚C) in a wizard DNA purification kit (Promega). Next, 50 μL 1 M NaOH were added to the eluant and the mixture incubated at room temperature for 5 minutes. After the pellet had been precipitated with 150 μL 100% isopropanol and washed with 70% ethanol, it was resuspended in 45 μL H_2O. Modified DNA was amplified in a total volume of 20 μL solution containing 1X PCR buffer, 1.0 mM $MgCl_2$, 100 ng of each primer, 0.2 mM dNTPs and 2.5 U Taq polymerase. PCR was performed in a thermal cycler for 35 cycles; each cycle consisted of denaturation at 94˚C for 1 minute, annealing at 60˚C for both methylated and unmethylated primers for 1 minute, extension at 72˚C for 1 minute and a final 5-minute extension at 72˚C. The PCR products were then loaded and electrophoresed on 3.5% agarose gel, stained with ethidium bromide and visualized under UV illumination.

CpG methylase (*Sss*I)-treated genomic DNA was the positive control for methylation-specific primers. DNA samples from the blood of healthy individuals, which were negative for *CDS1* methylation and positive for unmethylation, served as a positive control for unmethylated PCR. To ensure the specificity of *CDS1* methylation, unmodified genomic DNA samples from non-cancer parts and cancer of HCC patients were also carried out for negative controls.

Sequencing of methylation-specific PCR products. To calculate the numbers of methylated CpG sites, bisulfite-modified DNA was amplified by PCR with a primer set designed to detect both methylated and unmethylated promoter regions of the *CDS1* gene as follows: CDS-C-5' (forward) 5'-GCG TTC GTT GTA GGC GGT TT-3' (–220 to –201 of the transcriptional start site of the human *CDS1* gene) and CDS-C-3' (reverse) 5'-CAA CTT CCC CGA TAC CTC AA-3' (+29 to +10 of the transcriptional start site of the human *CDS1* gene). The amplification area corresponds to +29 to –220 of the transcriptional start site of the human *CDS1* gene. PCR was performed as described for methylation-specific PCR. Cloning reactions were carried out by the TOPO TA cloning kit version K2 (Invitrogen, Groningen, The Netherlands). Briefly, CDS-C PCR products were first purified and then 3' A-overhangs were added during incubation in 1 U Taq and 2.5 mM deoxyadenosine triphosphate (dATP) at 72˚C for 10 minutes. The mixture was then put on ice. The PCR products with A-overhangs were subsequently incubated with a vector at room temperature for 30 minutes and then put on ice. The reaction mixture was added to TOPO-competent cells and incubated on ice for 20 minutes. It was subsequently heated to 42˚C for 30 seconds to heat shock the competent cells, before immediate placement on ice. The mixture was then added to 250 μL SOC (2% bacto-tryptone, 0.5% bacto-yeast extract, 0.05% NaCl and 20 mM glucose) medium and shaken horizontally at 37˚C for 1 hour, before being plated on agar plates containing X-gal and 50 μg/mL ampicillin and incubation at 37˚C overnight. The next day, the colonies were picked and cultured overnight in Luria-Bertani (L13) medium containing 50 mg/mL ampicillin. Subsequently, the plasmid DNA was extracted from the competent cells by the QIApre Spin Miniprep Kit (Qiagen, Hilden, Germany) and digested with *Eco*RI to examine whether the plasmid DNA contained the correct insert. The plasmid DNA containing the correct insert was then sequenced with SP6 or T7 primers in an ABI Prism 310 Genetic Analyzer.

Statistics. Comparisons between the expression of *CDS1* in tumor and non-tumor tissues were made by a *t*-test run on SPSS for Windows Release 9.0 (SPSS, Chicago, IL, USA).

Results

Determination of CDS1 expression level by real-time quantitative RT-PCR. To investigate whether the expression of the *CDS1* gene was down-regulated in patients with HCC, 52 HCC and paired non-cancer tissues were analyzed by real-time quantitative RT-PCR. For internal control of the RNA, aminolevulinate-delta-synthase 1 (*ALAS1*), hypoxanthine phosphoribosyltransferase 1 (*HPRT1*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), hydroxymethylbilane synthase (*HMBS*), β-2-microglobulin (*B2M*), peptidylprolyl isomerase A (*PPIA*) and transferring receptor (*TFRC*) were tested. The results showed that the expression of *GAPDH* was most reliable. The amount of *CDS1* was normalized to the endogenous reference *GAPDH*. The normalized *CDS1* expression (ΔC_t) of HCC patients was then related to the ΔC_t of paired non-cancer tissues for their relative expression levels. The results showed that the expression levels of *CDS1* in HCC were significantly lower than those in paired non-cancer tissues. The relative expression levels ($2^{-\Delta\Delta Ct}$) of CDS1 for HCC were 1:13.75 of non-cancer tissues (Table II).

Table II. *Real-time quantitative RT-PCR and methylation analyses of the CDS1 gene in HCC.*

	Case no.	ΔC_t*	$\Delta\Delta C_t$	$2^{-\Delta\Delta C_t}$	Methylation rate	No. of CpG methylations
Tumor	52	1.09±0.70^{+}	3.78	1/13.75	73%$^{++}$	14^{+++}
Non-tumor	52	–2.69±0.66	–3.78	13.75/1	50%	9

*mean±SD; $^{+}p<0.01$; $^{++}p<0.05$; $^{+++}p<0.05$.

CDS1 gene expression was not down-regulated by mutations within coding region of the CDS1 gene. To elucidate the mechanism for the down-regulation of the *CDS1* gene, the mutations in the coding region were analyzed by direct sequencing. However, only one mutation resulting in an amino acid change (codon 66 GAT→GGT, Asp→Gly) and one polymorphism (intron 7 nt 104 G→A) were found in the *CDS1* gene (Figure 1). The allelic frequency of codon 66 GAT→GGT was 0.9%, and it was found in both tumor and non-tumor tissues. No difference in *CDS1* expression was found between the wild-type and these two mutations. These results suggest that mutations of *CDS1* in these regions are not responsible for the down-regulation of the *CDS1* gene in HCC tissues. Further, intron 7 nt 104 G→A polymorphism was used as a marker to test the LOH at the *CDS1* region, but no LOH change was found in 20 heterozygous cases. From these results, we suggest that the deletion of *CDS1* in HCC is not common.

Methylation inactivated CDS1 gene expression in hepatocellular carcinoma. To investigate whether the aberrant methylation of the CpG island was the mechanism for the down-regulation of the *CDS1* gene in HCC, the methylation status of the promoter area of the *CDS1* gene was analyzed. We designed two pairs of methylation-specific primers, designated CDS-M and CDS-U, to discriminate between methylated and unmethylated alleles and to discriminate between bisulfate-modified and unmodified DNA.

In Figure 2, four representative cases of the methylation status of *CDS1* are shown. They represent higher expression

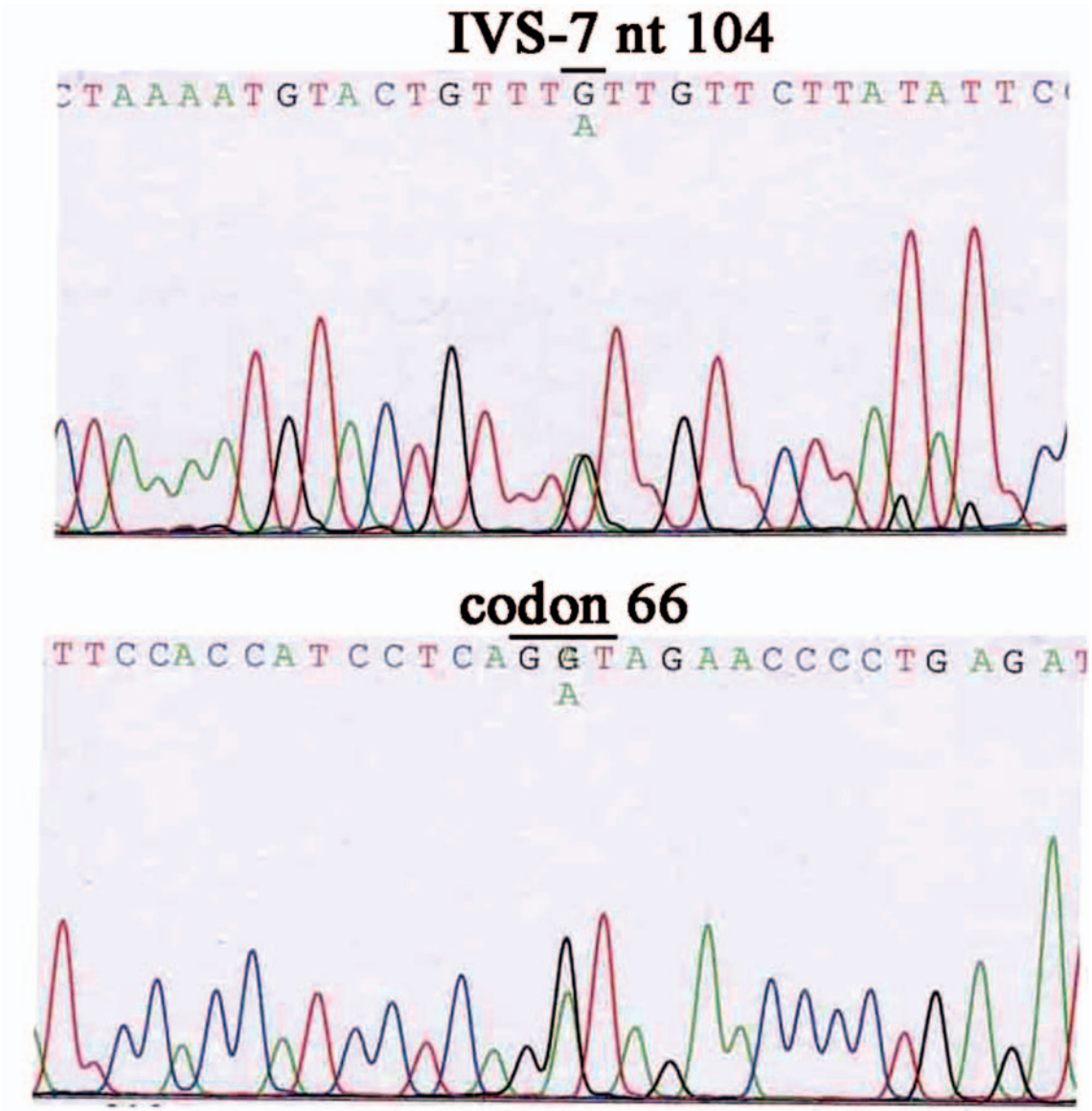

Figure 1. *Mutation analysis of the CDS1 gene revealed a mutation (codon 66 GAT→GGT, Asp→Gly) and a polymorphism (IVS-7 nt 104 G→A).*

in both cancer and paired non-cancer tissues (case 1), cancer tissue higher than paired non-cancer tissue (case 2), non-cancer tissue higher than cancer tissue (case 3) and lower expression in both cancer and non-cancer tissues (case 4), respectively (Figure 2A). The CDS-M and CDS-U primer sets are specific for methylated and unmethylated alleles, respectively; in addition, both primer sets are specific for bisulfite-modified DNA but not for unmodified genomic DNA (Figure 2B). The CDS-M and CDS-U primer sets both cover 22 CpG sites. The PCR products of these primer sets were direct-sequenced to calculate the numbers of methylated CpG sites (examples in Figure 3). As shown in Table II, methylation was observed in both non-cancer and cancer tissues. However, the methylated CpG frequencies of the *CDS1* gene of non-cancer tissues were lower than those of paired HCC tissues. In addition, most of the methylated non-cancer tissues carried partial methylated sites, whereas all sites were methylated in cancer tissues in most cases. On analysis of the promoter methylation of *CDS1* in three HCC cell lines, our results showed that the Mal-5 cell line expressed the promoter methylation. We also designed a universal primer that covers CpG sites which can detect both methylated and unmethylated alleles. In total, six cases of HCC were analyzed and the PCR products were subjected to subcloning analysis. Figure 4 shows the allelic patterns of methylated CpG islands of a representative case of HCC and paired non-cancer

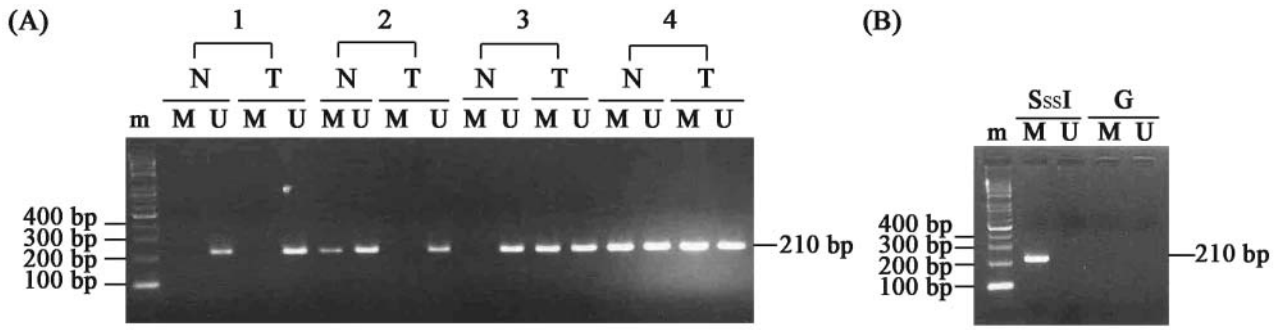

Figure 2. *Methylation-specific PCR of CDS1 gene for four representative cases. SssI is SssI-treated bisulfite-modified DNA of a healthy individual. This DNA was used as a positive control for methylation-specific primers. Genomic DNA from a HCC patient is unmodified (G). M and U indicate methyation-specific PCR using CDS-M and CDS-U primer sets, respectively. N and T indicate paired non-cancer tissue and cancer tissue, respectively. The m represents the 100-bp ladder DNA marker.*

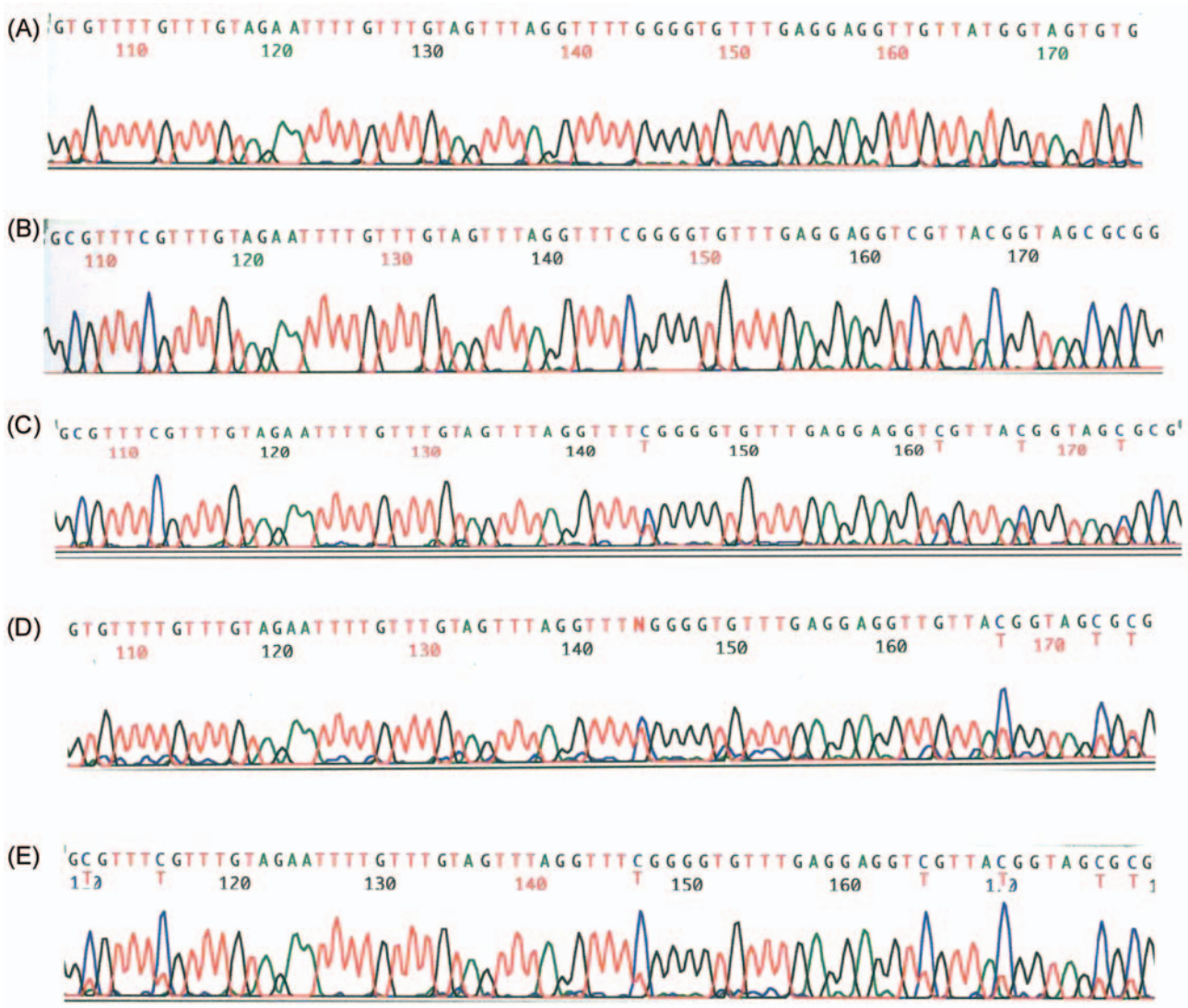

Figure 3. *Representative cases of methylation analysis of CpG islands at the promoter area of the CDS1 gene. The sequence corresponds to the transcriptional start site of the CDS1 gene. (A) Modified DNA of an unmethylated sample with all the cytosines converted to thymidine. (B) Modified DNA of a methylated sample with all the cytosines in the CpG remaining unchanged. (C-E) Modified DNA of partially methylated samples with part of the cytosine in CpG dinucleotides showing C/T heterozygosity.*

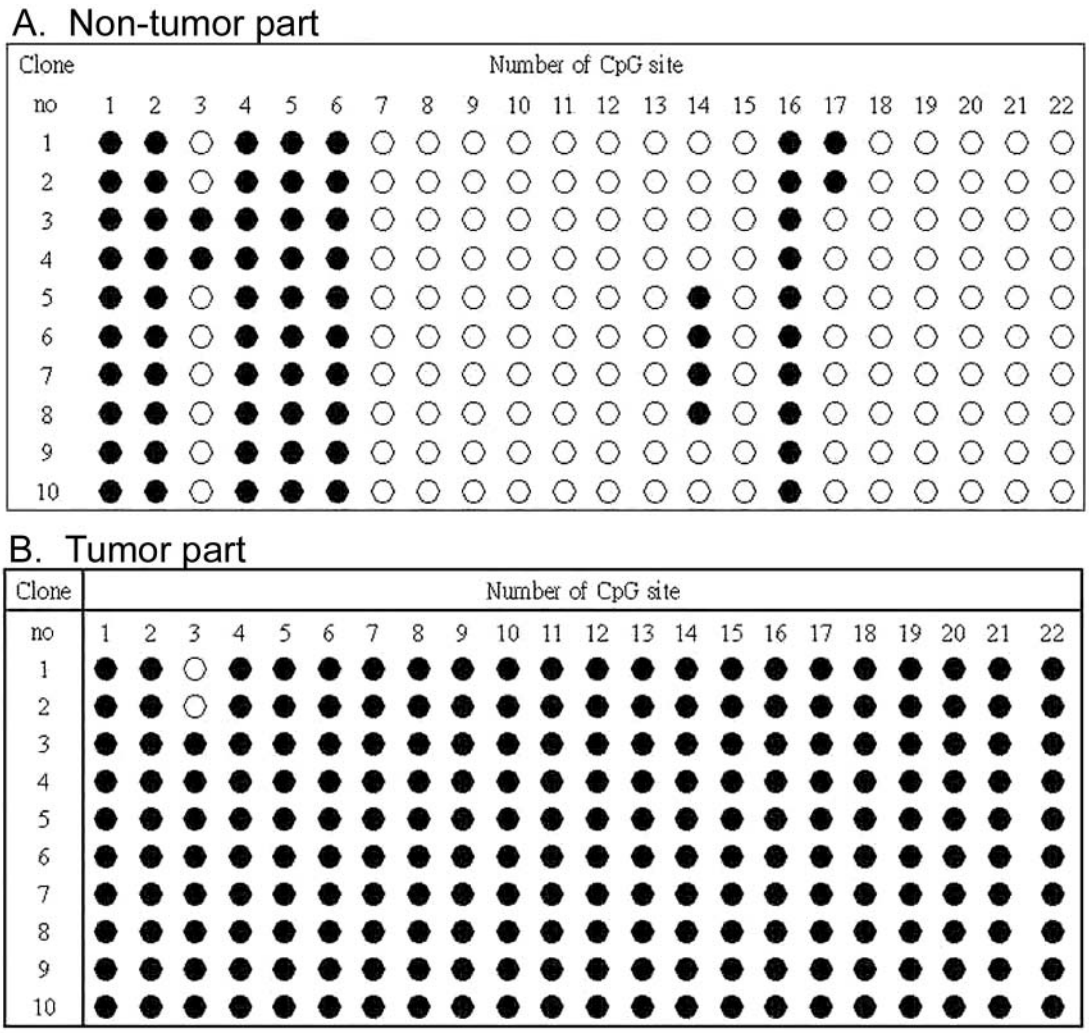

Figure 4. *Allelic patterns of CpG site methylation at the promoter area of the CDS1 gene. Methylation of the CDS1 CpG island located between positions +29 to –168, corresponding to the transcriptional starting site, was analyzed by genomic bisulfite sequence analysis. Ten clones were randomly related and sequenced. Methylated CpG sites are marked as filled circles (●) and unmethylated sites as open circles (○). The allelic patterns of the non-tumor part (A) or tumor part (B) of a representative HCC.*

tissues. In this representative case, several clones from the paired non-cancer tissue had fewer methylated CpG islands than the HCC tissue.

The methylation status of the *CDS1* promoter with the mRNA expression in each case were compared. Our results showed that 30 cases of cancer tissues having *CDS1* promoter methylation showed downexpression of *CDS1* mRNA, in comparison with the paired non-cancer tissues, but eight cases of cancer tissues having *CDS1* promoter methylation showed no differences in *CDS1* mRNA expression in comparison with the paired non-cancer tissues; 17 cases of non-cancer tissues with *CDS1* promoter methylation showed downexpression of *CDS1* mRNA in comparison with the paired cancer tissues, but nine cases of non-cancer tissues with *CDS1* promoter methylation showed no difference in *CDS1* mRNA expression with the paired cancer tissue. The detailed results are shown in Table III.

The HBV-status of the HCC cases was also compared with the expression and promoter methylation of *CDS1*. The results are shown in Table IV. Forty-eight out of 52 HCCs were HBV-related and there was a difference between the HBV status and the promoter methylation or mRNA expression of *CDS1*.

Discussion

Phosphatidic acid (PA) is a phospholipid involved in signal transduction and in glycerolipid biosynthesis. Newly-produced PA is a potential second messenger (17) with various candidate target proteins, including Raf-1 kinase (18), PKC-ξ (19), phosphatidylinositol 4-phosphate 5-kinase (20) and

Table III. *The relationship between the promoter methylation and mRNA expression of the CDS1 gene in 52 HCCs.*

			RNA expression
Tumor	Methylation*	30	↓
		0	↑
		8	-
	Unmethylation	12	↑
		2	-
Non-tumor	Methylation*	17	↓
		0	↑
		9	-
	Unmethylation	22	↑
		1	↓
		3	-

*3 cases had no methylation in their paired cancer tissues. *$p<0.05$.

Table IV. *The correlation of HBV status with CDS-1 promoter methylation and mRNA expression in HCC.*

HBV		Methylation*		mRNA expression+	
	p=48	p	37	↓	29
				-	8
		n	11	↑	10
				- or ↑	1
	n=4	p	1	↓	1
				-	0
		n	3	↑	2
				- or ↑	1

p: positive; n: negative; ↓: decrease; ↑: increase; -: no difference; *: $p<0.05$; +: $p<0.05$.

protein tyrosine-phosphatase (21). CDP-diacylglycerol synthase (CDS) or CTP (phosphatidate cytidylyltransferase; EC 2.7.7.7.41) catalyzes the conversion of PA to CDP-diacylglycerol (CDP-DAG), an important precursor for the synthesis of phosphatidylinositol, phosphatidylglycerol and cardiolipin. CDS is important in controlling the activity of the phosphoinositide cycle in signal transduction system (8-10).

Two *CDS* genes, *CDS1* and *CDS2*, are located at chromosomes 4q21 and 20p13, respectively. Chromosome 4q21 has been found to be deleted in HCC (22-25), hepatoblastoma (26), vulval and penile squamous cell carcinoma (27, 28) and esophageal adenocarcinoma tissue samples (29). A tumor suppressor gene (TSG) in this region is suspected, but it has yet to be identified. Based on the above evidence, we predict that *CDS1* may be the TSG in this region. *CDS1* gene expression was analyzed in HCC, and found to be significantly down-regulated. The intron 7 nt 104 polymorphism was used as a marker to test LOH in the *CDS1* region, but we were unable to find the deletion of the *CDS1* gene in 20 informed cases. From these results, we suggest that *CDS1* may not be the deleted gene in chromosome 4q21 in HCC. Because of the down-regulation of CDS1 in most cases of HCC in this study, we suggest that *CDS1* may play a role in the development of HCC.

Hypermethylation of promoter regions is an important mechanism in abolishing gene transcription in cancer. Numerous studies have reported the inactivation of TSGs by point mutation or deletion in the development of cancers. However, epigenetic silencing of TSG by promoter region hypermethylation is also common in human cancer. Aberrant methylation of normally unmethylated CpG islands has been associated with transcriptional inactivation of defined TSGs in human cancer (30, 31). In this situation, promoter region hypermethylation acts as an alternative to coding region mutations in eliminating TSG function. Here, the coding regions of the *CDS1* gene were first analyzed for mutations, but none were found. Therefore, the methylation of CpG sites in the promoter area of *CDS1* were analyzed by methylation-specific PCR and direct sequencing, and hypermethylation of *CDS1* was found at promoter CpG sites in HCC tissues. Most cancer tissues contained many more methylated CpG sites than paired non-cancer tissues; most also correlated well with *CDS1* gene expression. In the methylation study, three cases of non-cancer tissues showed methylation in the *CDS1* promoter, but no methylation was detected in the promoter in their paired cancer cells. This phenomenon has also been found in other cancer-related genes and suggests that a mechanism leading to deregulation in CpG-island methylation may be involved in the early carcinogenic process (32). In combination with the HBV-status, mutational analysis and methylation analysis, our data suggested that mutation or deletion are irrelevant to *CDS1* down-regulation; instead, the methylation of the CpG site at the promoter area is responsible for the down-regulation of the *CDS1* gene in patients with HCC; and the HBV-status is correlated with promoter methylation and expression of *CDS1*.

From these results, we suggest that the methylation of CpG sites of the *CDS1* gene may play an important role in the development of HCC.

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