

# Clinical utility of *PDSS2* expression to stratify patients at risk for recurrence of hepatocellular carcinoma

MITSURO KANDA, HIROYUKI SUGIMOTO, SHUJI NOMOTO, HISAHARU OYA, DAI SHIMIZU, HIDEKI TAKAMI, RYOJI HASHIMOTO, FUMINORI SONOHARA, YUKIYASU OKAMURA, SUGURU YAMADA, TSUTOMU FUJII, GORO NAKAYAMA, MASAHIKO KOIKE, MICHITAKA FUJIWARA and YASUHIRO KODERA

Department of Gastroenterological Surgery (Surgery II), Nagoya University Graduate School of Medicine, Nagoya, Japan

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**Abstract.** Identification of novel genetic and epigenetic alterations is required for optimal stratification of patients with hepatocellular carcinoma (HCC) at risk for recurrence and adverse prognosis. Coenzyme Q10 (CoQ10), which mediates apoptosis, is synthesized by prenyl diphosphate synthase subunit 2 (*PDSS2*). In the present study we evaluated the clinical significance and regulatory mechanisms of *PDSS2* expression in HCC. *PDSS2* expression levels and those of genes encoding potentially interacting proteins as well as the methylation status of the *PDSS2* promoter region were analyzed in HCC cell lines. *PDSS2* mRNA levels in 151 pairs of resected specimens were determined to evaluate the association of *PDSS2* expression and clinicopathological factors. The expression and distribution of *PDSS2* were determined using immunohistochemistry. *PDSS2* mRNA expression was decreased in six of nine HCC cell lines and significantly correlated with those of hepatocyte nuclear factor 4 $\alpha$ . *PDSS2* transcription in HCC cells with decreased *PDSS2* expression accompanying hypermethylation was reactivated after treating these cells with a methylation inhibitor. Mean expression levels of *PDSS2* mRNA relative to that of uninvolved liver diminished gradually in the order of chronic hepatitis to cirrhosis, and each was significantly higher than those of HCCs. *PDSS2* and *PDSS2* mRNA levels were consistent. Decreased *PDSS2* mRNA levels were detected in HCC tissues of 56 patients, correlated with shorter disease-specific survival, and was identified as an independent prognostic factor. *PDSS2* is a putative tumor suppressor, and promoter hypermethylation is a key regulatory mechanism in HCC. Decreased levels of *PDSS2* mRNA expression may represent a novel biomarker of HCC.

## Introduction

Hepatocellular carcinoma (HCC) is one of the most lethal cancers worldwide and is the main cause of death among cirrhotic patients (1-3). Patients diagnosed with HCC have a poor prognosis because of the aggressive nature of the disease (4,5), and surgical resection or local ablation therapy is effective only at an early stage (6). Furthermore, ~70% of these patients develop recurrent tumors within five years after curative surgery (7). Recurrence of HCC, including multicentric hepatocarcinogenesis and intrahepatic metastasis, is a key prognostic factor, but it is difficult to distinguish patients at high risk for recurrence and subsequent adverse prognosis using only clinical staging systems comprising tumor characteristics and liver function (1,8). Therefore, a novel approach for predicting progression and recurrence of HCC is urgently required.

Liver damage and the increased incidence of HCC (chronic viral hepatitis B and C, alcohol consumption and aflatoxin) have multiple causes (5,9,10). Furthermore, as with other malignancies, the initiation of HCC is a multistep process, and because it is characterized by high molecular variability, clinical management requires a more complex approach (11).

Although recent research along with the development of new genomic technologies establishes that the development and progression of HCC are caused by the accumulation of genetic and epigenetic alterations (12-14), the detailed underlying mechanisms have not been determined. Therefore, identifying molecular markers for HCC, particularly those that may predict recurrence, is important, because stratification of patients at risk for recurrence facilitates individualized management, including intensive surveillance and aggressive adjuvant therapy for high-risk patients.

Prenyl diphosphate synthase subunit 2 (*PDSS2*) was identified in 2005 (15), it encodes the second subunit of prenyl diphosphate synthase, which is an essential enzyme involved in the biosynthesis of coenzyme Q10 (CoQ10), and *PDSS2* determines the side-chain length of mammalian ubiquinones (16). CoQ10 is synthesized from mevalonic acid in the liver and plays a vital role in the mitochondrial respiratory chain, pyrimidine nucleoside biosynthesis and the modulation of cell apoptosis (17). Aberrant expression of *PDSS2* in the liver may cause DNA damage and disrupt the cell cycle through inhibi-

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*Correspondence to:* Dr Mitsuro Kanda, Department of Gastroenterological Surgery (Surgery II), Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan  
E-mail: m-kanda@med.nagoya-u.ac.jp

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tion of CoQ10 synthesis, leading to initiation and progression of HCC (18,19). Furthermore, chronic inflammation caused by hepatitis virus infection might affect *PDSS2* expression. Although evidence indicates that *PDSS2* suppresses the development of malignant melanoma and lung cancer (16,20), the clinical significance and regulatory mechanisms of *PDSS2* expression in HCC remain undefined.

Therefore, we attempted to answer these questions in the present study by analyzing *PDSS2* expression in HCC to identify novel, clinically significant biomarkers for progression and recurrence of HCC. To the best of our knowledge, this is the first report to determine *PDSS2* expression levels in HCC.

## Materials and methods

**Sample collection.** Nine HCC cell lines (Hep3B, HepG2, HLE, HLF, HuH1, HuH2, HuH7, PLC/PRF/5 and SK-Hep1) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were maintained as previously described (21). Primary HCC and non-cancerous tissues were collected from 151 patients who underwent liver resection for HCC at Nagoya University Hospital between January 1998 and July 2012. Clinicopathological data were collected from medical records. Specimens were classified histologically according to the 7th Edition of the Union for International Cancer Control classification (22).

Tissue samples were immediately flash-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . RNA was extracted from  $\sim 5\text{ mm}^2$  diameter tumor samples without detectable necrotic areas comprising  $>80\%$  tumor cells. The corresponding non-cancerous liver tissue samples that lacked regenerative or dysplastic nodules were collected from the same patient that were  $>2\text{ cm}$  distant from the edge of the tumor. The median duration of patient follow-up was 37.9 months (range, 0.37–147 months). Postoperative follow-up included physical examinations, measurement of serum tumor markers every three months, and enhanced computed tomography scans every six months. Treatment after recurrence was generally selected from one of the options as follows: surgery, radiofrequency ablation, transcatheter arterial chemoembolization, and chemotherapy, according to tumor status and liver function. Enrollees granted written informed consent for the use of clinical samples and data as required by the Institutional Review Board of Nagoya University, Japan.

**Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR).** *PDSS2* mRNA levels were determined using qRT-PCR. Total RNA ( $10\text{ }\mu\text{g}$ ) was isolated from 9 HCC cell lines, 151 primary HCCs and adjacent non-cancerous tissues, and was used as a template for cDNA synthesis. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA (TaqMan, *GAPDH* control reagents; Applied Biosystems, Foster City, CA, USA) was quantified in each sample for standardization. Quantitative real-time RT-PCR was performed using the SYBR<sup>®</sup> Green PCR Core Reagents kit (Applied Biosystems) as follows: one cycle at  $95^{\circ}\text{C}$  for 10 min, 40 cycles at  $95^{\circ}\text{C}$  for 5 sec, and  $60^{\circ}\text{C}$  for 60 sec. Real-time detection of the SYBR<sup>®</sup> Green fluorescence was conducted using an ABI StepOnePlus<sup>™</sup> Real-Time PCR System (Applied Biosystems). Triplicate samples of 9 HCC cell lines and 151 clinical samples

were analyzed. Samples without templates served as negative controls. The expression level of each sample is shown as the value of the *PDSS2* amplicon divided by that of *GAPDH* (23). The primer sequences are listed in Table I. *PDSS2* mRNA levels were considered downregulated in tumor tissues when they were  $<50\%$  compared with those of the corresponding non-cancerous tissues.

**Analysis of the promoter region of *PDSS2*.** The nucleotide sequence of the *PDSS2* promoter region was analyzed to determine the presence or absence of CpG islands defined as follows: at least a 200-bp region of DNA with a high HCC content ( $>50\%$ ) and an Observed CpG/Expected CpG ratio  $\geq 0.6$  (24,25). CpG Island Searcher software (<http://cpgislands.usc.edu/>) was employed to determine the locations of CpG islands (26).

**Methylation-specific PCR (MSP) and bisulfite sequence analysis.** *PDSS2* possesses a CpG island near its promoter region, and we hypothesized that aberrant methylation regulates *PDSS2* transcription in HCC. DNA samples from nine HCC cell lines treated with bisulfite were subjected to MSP. Genomic bisulfite-treated DNA from HCC cell lines was sequenced to ascertain whether the MSP amplification was reliable. The primer sequences used for MSP and bisulfite sequencing are listed in Table I. Bisulfite treatment and the sequencing procedure were performed as reported (27).

**5-Aza-2'-deoxycytidine (5-aza-dC) treatment.** To assess the relation of promoter hypermethylation to *PDSS2* transcription, HCC cells ( $1.5 \times 10^6$ ) were treated with 5-aza-dC (Sigma-Aldrich, St. Louis, MO, USA) to inhibit DNA methylation and cultured for 6 days with medium changes on days 1, 3 and 5. RNA was extracted, and RT-PCR was performed as previously described (27).

**Expression of genes encoding proteins potentially associated with *PDSS2*.** The expression levels of hepatocyte nuclear factor 4 $\alpha$  (HNF4 $\alpha$ ) and caudal-type homeobox transcription factor 2 (CDX2), which may associate with *PDSS2* (20,28) were determined in HCC cell lines using qRT-PCR. The sequences of primers specific for HNF4 $\alpha$  and CDX2 are listed in Table I.

**Immunohistochemistry (IHC).** IHC analysis of the localization of *PDSS2* was performed using a mouse monoclonal antibody against *PDSS2* (ab119768; Abcam, Cambridge, UK) diluted 1:150 in antibody diluent (Dako, Glostrup, Denmark) to probe 30 representative sections of well-preserved HCC tissue previously described (2). Staining patterns were compared between HCCs and the corresponding normal adjacent tissues. To avoid subjectivity, the specimens were randomized and coded before analysis by two independent observers who were unaware of the status of the samples. Each observer evaluated all specimens at least twice to minimize intraobserver variation (29).

**Statistical analysis.** Correlations between the levels of *PDSS2* mRNA with those of HNF4 $\alpha$  and CDX2 were analyzed using the Spearman rank correlation test. Relative levels of mRNA expression (*PDSS2*/*GAPDH*) between HCC and non-

Table I. Primers and annealing temperatures.

Gene	Experiment	Type	Sequence (5'-3')	Product size (bp)	Annealing temperature (°C)
<i>PDSS2</i>	qRT-PCR	Forward	GAATCAGGTAGTGTCTCAGAGG	181	60
		Reverse	GAGGCTATTCCAGCTGTCATG		
	MSP methylated	Forward	TCGAAGTTGGATTCTGAGGAT	263	62
		Reverse	AAACGTCGAACGAAAACACC		
	MSP unmethylated	Forward	GGTTGGTGGTGATAGTGATA	195	56
		Reverse	CAACAAATAATCCCTCTACAC		
	Bisulfite sequencing	Forward	TGTTTGGTTGGGTTTTGAGG	152	58
		Reverse	CACCAACCCCTAACAATAAC		
<i>HNF4<math>\alpha</math></i>	qRT-PCR	Forward	CGTGGTGGACAAAGACAAGA	128	60
		Reverse	CATAGCTTGACCTTCGAGTGC		
<i>CDX2</i>	qRT-PCR	Forward	GGAACCTGTGCGAGTGGAT	128	60
		Reverse	GAAACTCCTTCTCCAGCTCC		
<i>GAPDH</i>	qRT-PCR	Forward	GAAGGTGAAGGTCGGAGTC	226	60
		Probe	CAAGCTTCCCGTTCTCAGCC		
		Reverse	GAAGATGGTGATGGGATTTTC		

*PDSS2*, prenyl diphosphate synthase subunit 2; *HNF4 $\alpha$* , hepatocyte nuclear factor 4 $\alpha$ ; *CDX2*, caudal-related homeobox transcription factor 2; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; qRT-PCR, quantitative real-time reverse-transcription polymerase chain reaction; MSP, methylation specific PCR; bp, base pair.

cancerous tissues were analyzed using the Mann-Whitney U test. The  $\chi^2$  test was used to analyze the significance of the association between the expression and methylation status of *PDSS2* and clinicopathological parameters. Disease-specific and disease-free survival rates were calculated using the Kaplan-Meier method, and the difference in survival curves was analyzed using the log-rank test. We performed multivariate regression analysis using the Cox proportional hazards model to detect prognostic factors, and variables with  $P < 0.05$  were entered into the final model. All statistical analyses were performed using JMP 10 software (SAS Institute, Inc., Cary, NC, USA).  $P < 0.05$  was considered statistically significant.

## Results

**Identification of a CpG island in the *PDSS2* promoter region.** A CpG island was identified in the *PDSS2* promoter region (Fig. 1A), leading to the hypothesis that hypermethylation of the CpG islands regulates the expression of *PDSS2* in HCC.

***PDSS2* mRNA expression and regulatory mechanisms in HCC cell lines.** Significant decrease in *PDSS2* mRNA levels was detected in 6 (67%) of 9 HCC cell lines compared with the mean expression level in 151 non-cancerous liver tissues (Fig. 1B). Hypermethylation of the *PDSS2* promoter was detected in Hep3B, HuH2, HuH7 and SK-Hep1 cells (Fig. 1B). To determine whether hypermethylation of the *PDSS2* promoter inhibited expression, *PDSS2* mRNA expression

levels were compared before and after treatment with the methylation inhibitor 5-aza-dC. *PDSS2* mRNA levels were restored in cells with downregulated *PDSS2* expression accompanying hypermethylation after 5-aza-dC treatment (Fig. 1B), indicating that promoter hypermethylation inhibited *PDSS2* transcription in HCC.

**Expression of genes encoding proteins potentially associated with *PDSS2* in HCC cell lines.** The relative mRNA expression levels of *PDSS2*, *HNF4 $\alpha$*  and *CDX2* in HCC cell lines are shown in Fig. 2A. *PDSS2* expression levels significantly correlated with those of *HNF4 $\alpha$*  (Fig. 2B).

**Patient characteristics.** The mean age of the 151 patients was  $64.6 \pm 9.7$  years (range, 34-84 years), the male to female ratio was 5:1, and there were 37 and 84 patients with hepatitis B and C virus infections, respectively. Of the patients without HCC, 10, 87 and 54 presented with normal liver function, chronic hepatitis or cirrhosis, respectively. We classified 140 and 11 patients as Child-Pugh class A or B, respectively.

**Expression levels of *PDSS2* mRNA and protein in resected tissues.** The mean level of *PDSS2* mRNA compared with that of non-cancerous liver diminished gradually in the order of normal liver, chronic hepatitis and cirrhosis, indicating that chronic inflammation and fibrosis of non-cancerous liver decreased *PDSS2* expression (Fig. 3A). In contrast, the type of hepatitis virus infection (hepatitis virus B, C or none) did not influence *PDSS2* expression in non-cancerous liver tissues.

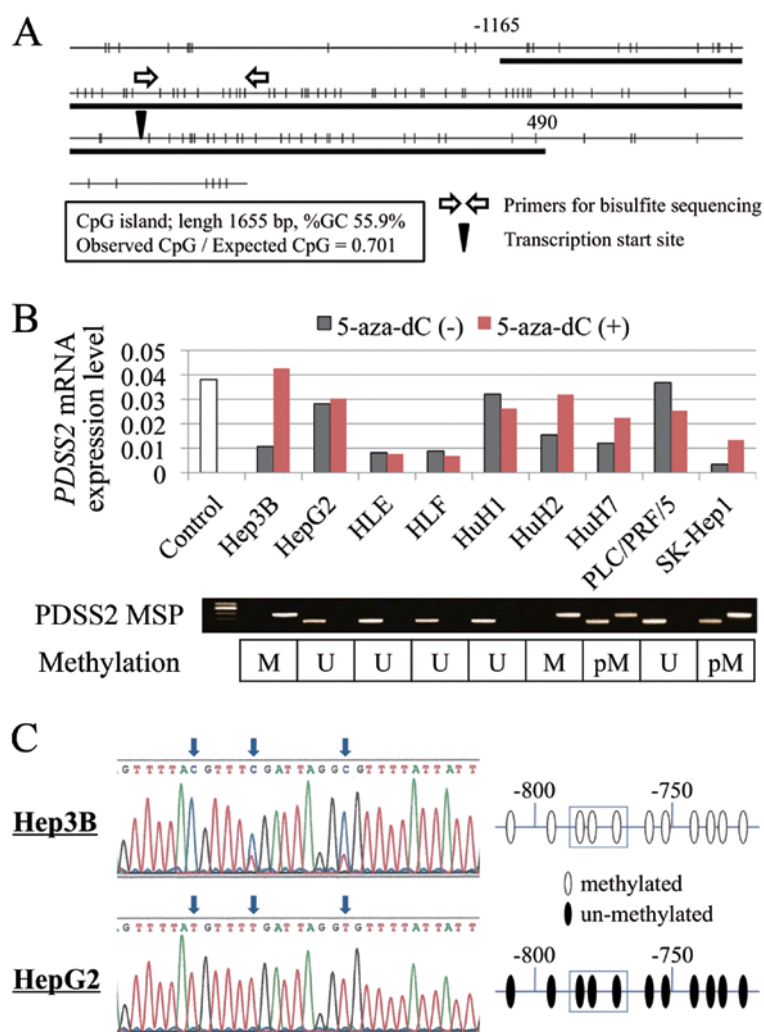


Figure 1. (A) A CpG island was detected near the PDSS2 transcription initiation site, extending upstream into the promoter region. (B) Bar graphs indicate PDSS2 mRNA expression in HCC cell lines before or after 5-aza-dC treatment. The methylation status of the PDSS2 promoter is shown in the box. M, methylated; pM, partially methylated; U, unmethylated. (C) Representative results of bisulfite sequence analysis. All CpG sites in Hep3B cells were retained as CG and those of HepG2 cells were converted to TG.

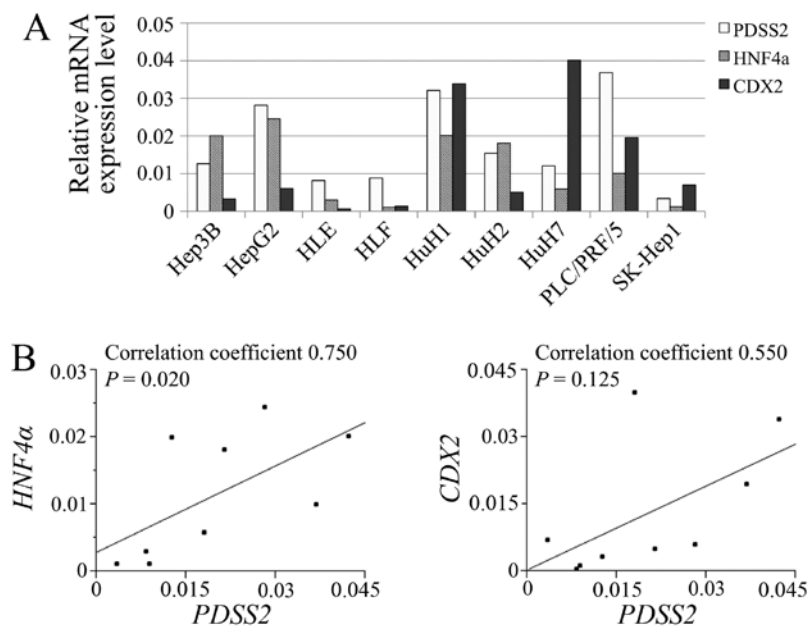


Figure 2. (A) Analysis of PDSS2, HNF4a and CDX2 mRNA expression in HCC cell lines. (B) Comparison of mRNA expression levels between PDSS2, HNF4a and CDX2.

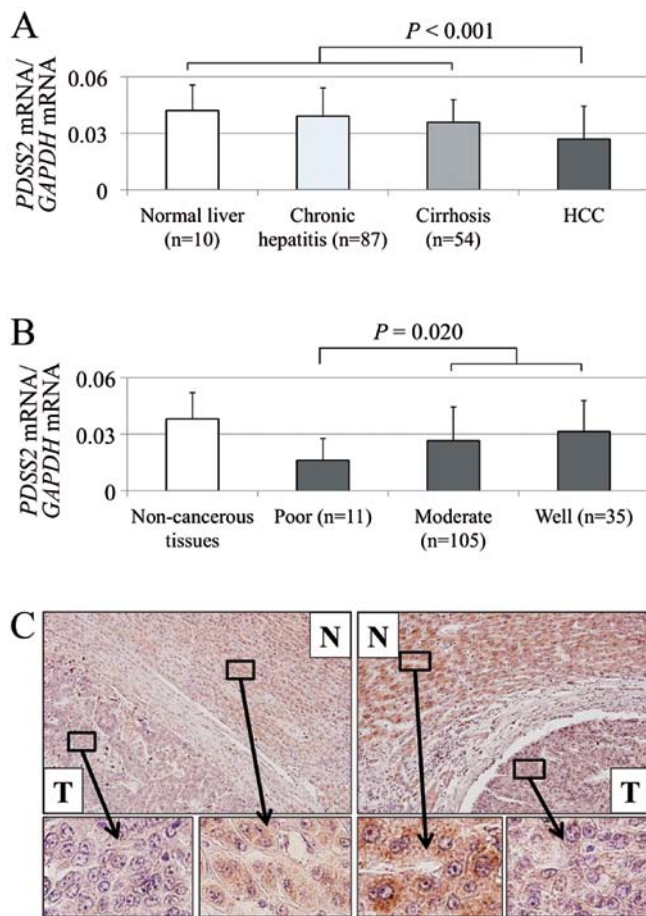


Figure 3. Analysis of the expression of *PDSS2* in clinical specimens. (A) The mean level of *PDSS2* mRNA expression was lower in HCC tissues compared with the corresponding non-cancerous tissues categorized by background uninvolved liver status. (B) Comparison of *PDSS2* mRNA expression level among HCCs categorized by tumor differentiation. (C) Representative IHC data. *PDSS2* was expressed at lower levels compared with non-cancerous adjacent tissue (magnified at x100 and x400). N, non-cancerous tissue; T, tumor tissue.

*PDSS2* mRNA expression was decreased in HCC tissues of 122 (81%) of 151 patients compared with that of the corresponding non-cancerous tissues. The mean expression level of *PDSS2* mRNA was significantly lower in HCC tissues compared with that of the corresponding non-cancerous tissues ( $P < 0.001$ ; Fig. 3A). Moreover, poorly differentiated tumor cells expressed relatively lower levels of *PDSS2* mRNA (Fig. 3B).

The expression of *PDSS2* was analyzed using IHC. Representative sections with reduced *PDSS2* staining in HCC tissues are shown in Fig. 3C. The overall staining intensities of 30 samples were consistent with mRNA levels detected using qRT-PCR.

**Prognostic implications of *PDSS2* mRNA expression levels.** Fifty-six (37%) of 151 patients were categorized with decreased *PDSS2* mRNA levels in HCC tissues compared with non-cancerous tissues. The disease-specific survival rate of patients with HCC with decreased *PDSS2* mRNA was significantly lower compared with those without this factor (5-year survival rates, 51 and 74%, respectively,  $P = 0.001$ ; Fig. 4A). Decreased *PDSS2* mRNA expression in patients with HCCs was significantly associated with uninvolved liver status, preoperative

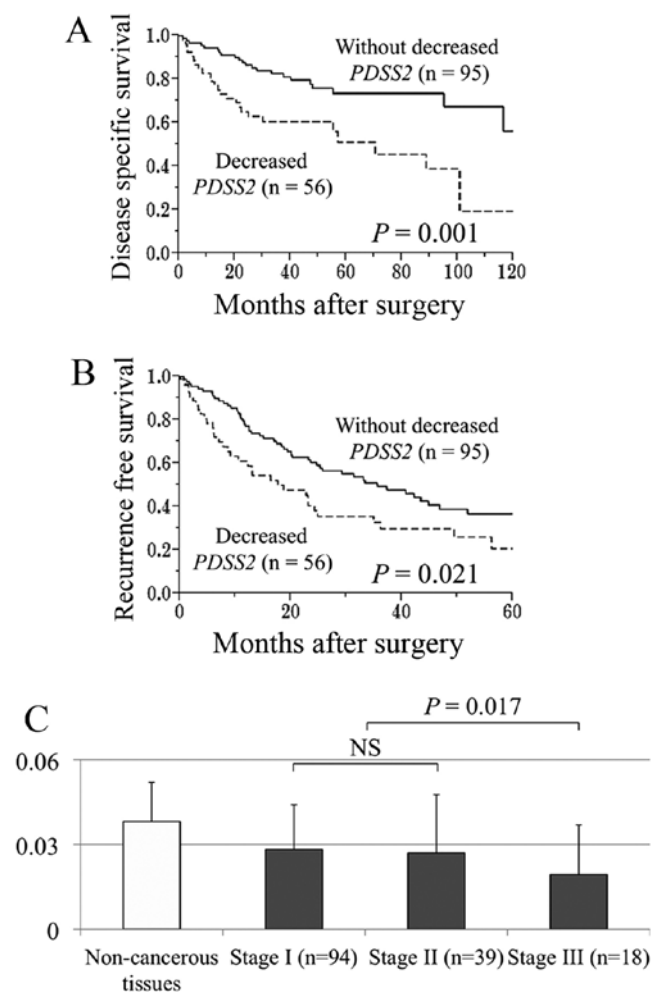


Figure 4. Prognostic significance of *PDSS2* mRNA expression in patients with HCC. (A) Disease-specific survival of patients with decreased *PDSS2* mRNA in HCC was significantly shorter than those without. (B) Recurrence-free survival of patients with decreased *PDSS2* mRNA was significantly shorter than those without. (C) *PDSS2* expression levels in each UICC stage.

serum  $\alpha$ -fetoprotein  $>20$  ng/ml, tumor size  $\geq 3.0$  cm, tumor differentiation (moderate to poor), serosal infiltration, septum formation and advanced UICC stage (Table II). Multivariate analysis identified decreased *PDSS2* mRNA expression as an independent prognostic factor (hazard ratio 2.45, 95% confidence interval 1.27-4.80,  $P = 0.008$ ; Table III). Patients with HCC with decreased *PDSS2* mRNA levels experienced significantly earlier recurrences compared with those without (2-year recurrence-free survival rates, 38 and 59%, respectively,  $P = 0.021$ ; Fig. 4B). A stepwise decrease of *PDSS2* mRNA expression in patients with HCC correlated with UICC stage (Fig. 4C).

## Discussion

In the present study, our data support the role of *PDSS2* as a suppressor of HCC. *PDSS2* mRNA was differentially expressed by HCC cell lines and was decreased in 81% of the HCC tissues. Hypermethylation of the *PDSS2* promoter was detected in four HCC cell types that expressed low levels of *PDSS2* mRNA. Moreover, *PDSS2* mRNA synthesis was reactivated after demethylation, indicating that promoter

Table II. Association between expression levels of *PDSS2* mRNA and clinicopathological parameters in 151 patients with hepatocellular carcinoma (HCC).

Clinicopathological parameters	Decreased <i>PDSS2</i> in HCCs (n=56)	Others (n=95)	P-value
Age (years)			
<65	26	41	0.696
≥65	30	54	
Gender			
Male	50	76	0.128
Female	6	19	
Background liver			
Normal liver	1	9	0.046 <sup>a</sup>
Chronic hepatitis	38	49	
Cirrhosis	17	37	
Pugh-Child's classification			
A	52	88	0.959
B	4	7	
Hepatitis virus			
Absent	13	17	0.551
HBV	15	22	
HCV	28	56	
AFP (ng/ml)			
≤20	18	63	<0.001 <sup>a</sup>
>20	38	32	
PIVKA II (mAU/ml)			
≤40	16	42	0.054
>40	40	53	
Tumor multiplicity			
Solitary	43	74	0.875
Multiple	13	21	
Tumor size (cm)			
<3.0	12	35	0.045 <sup>a</sup>
≥3.0	44	60	
Differentiation			
Well	7	28	0.014 <sup>a</sup>
Moderate to poor	49	67	
Growth type			
Expansive growth	43	84	0.063
Invasive growth	13	11	
Serosal infiltration			
Absent	35	79	0.005 <sup>a</sup>
Present	21	16	
Formation of capsule			
Absent	17	30	0.875
Present	39	65	
Infiltration to capsule			
Absent	24	44	0.680
Present	32	51	

Table II. Continued.

Clinicopathological parameters	Decreased <i>PDSS2</i> in HCCs (n=56)	Others (n=95)	P-value
Septum formation			
Absent	13	40	0.017 <sup>a</sup>
Present	43	55	
Vascular invasion			
Absent	33	81	<0.001 <sup>a</sup>
Present	23	14	
UICC pathological stage			
I	27	67	0.014 <sup>a</sup>
II	18	21	
III	11	7	

<sup>a</sup>Statistically significant difference (P<0.05). HBV, hepatitis B virus; HCV, hepatitis C virus; AFP,  $\alpha$ -fetoprotein; PIVKA, protein induced by vitamin K antagonists; UICC, Union for International Cancer Control.

hypermethylation regulated *PDSS2* transcription. To the best of our knowledge, the present study is the first to show a correlation between the expression and methylation status of *PDSS2*. However, decreased transcription of *PDSS2* was detected in some HCC cells without hypermethylation of the *PDSS2* promoter. Because *PDSS2* is located within chromosome 6q16.3-21, a site of frequent loss of heterozygosity (LOH) in HCC (30,31), we consider LOH as a possible alternative factor leading to dysregulation.

Recent *in silico* pathway analysis suggests that *PDSS2* interacts with HNF4 $\alpha$ , a nuclear transcription factor that acts as a tumor suppressor and regulates the expression of many genes involved in cell growth and proliferation (20,32). CDX2 is a tumor suppressor of various malignancies and interacts with HNF4 $\alpha$  (28,33). Accordingly, the correlations of mRNA expression between *PDSS2*, HNF4 $\alpha$  and CDX2 were evaluated, and we found that the expression of *PDSS2* correlated positively with that of HNF4 $\alpha$ . These findings support the function of *PDSS2* as a suppressor of HCC, and further pathway analysis will be required to support this conclusion.

*PDSSs* are heterotetrameric enzymes comprising subunits encoded by *PDSS1* (10p12.1) and *PDSS2* (15,16). In the absence of prenyl diphosphate synthase activity, CoQ10 is not synthesized (15). Therefore, decreased expression of *PDSS2* in the liver tissues may lead to reduced synthesis of CoQ10 by hepatocytes, resulting in the inhibition of tumor suppressors (17,18).

Similar to patients with malignant melanoma and lung cancer, most patients with HCC harbored decreased levels of *PDSS2* mRNA in HCC tissues, and their mean level of *PDSS2* expression was significantly decreased in HCC tissues compared with non-cancerous liver tissues (16,20). Notably, we show here a stepwise decrease of *PDSS2* expression accom-

Table III. Prognostic factors of 151 patients with hepatocellular carcinoma (HCC).

Variables	n	Univariate analysis			Multivariable analysis		
		Hazard ratio	95% CI	P-value	Hazard ratio	95% CI	P-value
Age ( $\geq 65$ years)	84	1.92	1.07-3.57	0.030 <sup>a</sup>	1.70	0.92-3.25	0.090
Gender (male)	126	1.27	0.60-3.13	0.553			
Background liver (cirrhosis)	54	1.58	0.88-2.81	0.123			
Pugh-Child's classification (B)	11	0.93	0.28-2.32	0.889			
AFP ( $>20$ ng/ml)	70	1.90	1.07-3.42	0.029 <sup>a</sup>	1.09	0.57-2.10	0.785
PIVKA II ( $>40$ mAU/ml)	93	2.10	1.14-4.07	0.016 <sup>a</sup>	1.20	0.62-2.49	0.595
Tumor multiplicity (multiple)	34	2.09	1.11-3.76	0.023 <sup>a</sup>	1.80	0.92-3.41	0.085
Tumor size ( $\geq 3.0$ cm)	104	2.20	1.13-4.71	0.020 <sup>a</sup>	1.40	0.63-3.43	0.418
Tumor differentiation (well)	35	0.55	0.25-1.10	0.095			
Growth type (invasive growth)	24	1.44	0.69-2.76	0.318			
Serosal infiltration	37	2.51	1.32-4.61	0.006 <sup>a</sup>	1.14	0.56-2.25	0.712
Formation of capsule	104	1.05	0.57-2.02	0.884			
Infiltration to capsule	83	1.20	0.67-2.18	0.537			
Septum formation	98	0.87	0.49-1.60	0.651			
Vascular invasion	37	3.40	1.87-6.07	$<0.001^a$	1.85	0.94-3.66	0.076
Margin status (positive)	28	2.64	1.42-4.73	0.003 <sup>a</sup>	2.60	1.36-4.84	0.005 <sup>a</sup>
Decreased <i>PDSS2</i> in HCC	56	2.52	1.41-4.52	0.002 <sup>a</sup>	2.45	1.27-4.80	0.008 <sup>a</sup>

<sup>a</sup>Statistically significant ( $P < 0.05$ ). CI, confidence interval; AFP,  $\alpha$ -fetoprotein; PIVKA, protein induced by vitamin K antagonists. Univariate analysis was performed using the log-rank test. Multivariate analysis was performed using the Cox proportional hazards model.

panied with chronic inflammation and fibrosis of uninvolved liver. Moreover, our data link the level of *PDSS2* expression to tumor differentiation. Taken together, these results suggest that *PDSS2* plays a role in suppressing multistep hepatocarcinogenesis.

Because the IHC and qRT-PCR data were consistent, we used the latter to assess the prognostic significance of *PDSS2* mRNA levels in a quantitative manner (24,29). We found that decreased *PDSS2* mRNA expression in HCCs was significantly associated with more aggressive tumor features, including elevated preoperative serum  $\alpha$ -fetoprotein, larger tumor size and serosal infiltration, and therefore, was identified as an independent prognostic factor. Moreover, patients with decreased *PDSS2* mRNA levels in their tumor tissues experienced significantly earlier recurrence after curative hepatectomy. Furthermore, the degree of the decrease in *PDSS2* expression correlated with UICC stage, indicating that the level of *PDSS2* expression reflects the severity of the malignant phenotype of HCC.

Our evidence that *PDSS2* act as a tumor suppressor is as follows: i) decreased expression of *PDSS2* was frequently detected and the mean level of *PDSS2* expression was significantly lower in HCC tissues, and ii) decreased expression of *PDSS2* was associated with shorter recurrence free survival and subsequent poor prognosis. *PDSS2* expression levels in biopsy or resected tissues may be useful for the prediction of recurrence and poor prognosis, which will facilitate efforts to devise an efficacious therapeutic strategy.

Our data support the conclusion that *PDSS2* acts as a tumor suppressor and that its expression is regulated by promoter hypermethylation in HCC. Moreover, decreased expression of *PDSS2* mRNA may represent a novel biomarker for predicting the progression and recurrence of HCC.

## References

- Minguez B and Lachenmayer A: Diagnostic and prognostic molecular markers in hepatocellular carcinoma. *Dis Markers* 31: 181-190, 2011.
- Kanda M, Nomoto S, Oya H, *et al*: Downregulation of DENND2D by promoter hypermethylation is associated with early recurrence of hepatocellular carcinoma. *Int J Oncol* 44: 44-52, 2014.
- Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics. *CA Cancer J Clin* 61: 69-90, 2011.
- El-Serag HB and Rudolph KL: Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. *Gastroenterology* 132: 2557-2576, 2007.
- Shiraha H, Yamamoto K and Namba M: Human hepatocyte carcinogenesis (Review). *Int J Oncol* 42: 1133-1138, 2013.
- El-Serag HB: Hepatocellular carcinoma: recent trends in the United States. *Gastroenterology* 127: S27-S34, 2004.
- Kanda M, Nomoto S, Nishikawa Y, *et al*: Correlations of the expression of vascular endothelial growth factor B and its isoforms in hepatocellular carcinoma with clinic-pathological parameters. *J Surg Oncol* 98: 190-196, 2008.
- Llovet JM, Burroughs A and Bruix J: Hepatocellular carcinoma. *Lancet* 362: 1907-1917, 2003.
- Kanda M, Nomoto S, Okamura Y, *et al*: Promoter hypermethylation of fibulin 1 gene is associated with tumor progression in hepatocellular carcinoma. *Mol Carcinog* 50: 571-579, 2011.

10. El-Serag HB and Mason AC: Rising incidence of hepatocellular carcinoma in the United States. *N Engl J Med* 340: 745-750, 1999.
11. Villanueva A, Newell P, Chiang DY, Friedman SL and Llovet JM: Genomics and signaling pathways in hepatocellular carcinoma. *Semin Liver Dis* 27: 55-76, 2007.
12. Miki D, Ochi H, Hayes CN, Aikata H and Chayama K: Hepatocellular carcinoma: towards personalized medicine. *Cancer Sci* 103: 846-850, 2012.
13. Kanda M, Nomoto S, Okamura Y, *et al*: Detection of metallothionein 1G as a methylated tumor suppressor gene in human hepatocellular carcinoma using a novel method of double combination array analysis. *Int J Oncol* 35: 477-483, 2009.
14. Herath NI, Leggett BA and MacDonald GA: Review of genetic and epigenetic alterations in hepatocarcinogenesis. *J Gastroenterol Hepatol* 21: 15-21, 2006.
15. Saiki R, Nagata A, Kainou T, Matsuda H and Kawamukai M: Characterization of solanesyl and decaprenyl diphosphate synthases in mice and humans. *FEBS J* 272: 5606-5622, 2005.
16. Fung JM, Smith R, Brown MA, *et al*: Identification and characterization of a novel melanoma tumor suppressor gene on human chromosome 6q21. *Clin Cancer Res* 15: 797-803, 2009.
17. Turunen M, Olsson J and Dallner G: Metabolism and function of coenzyme Q. *Biochim Biophys Acta* 1660: 171-199, 2004.
18. Quinzii CM, DiMauro S and Hirano M: Human coenzyme Q10 deficiency. *Neurochem Res* 32: 723-727, 2007.
19. DiMauro S, Quinzii CM and Hirano M: Mutations in coenzyme Q10 biosynthetic genes. *J Clin Invest* 117: 587-589, 2007.
20. Chen P, Yu J, Knecht J and Chen Q: Decrease of PDSS2 expression, a novel tumor suppressor, in non-small cell lung cancer. *Cancer Epidemiol* 37: 166-171, 2013.
21. Takami H, Kanda M, Oya H, *et al*: Evaluation of MAGE-D4 expression in hepatocellular carcinoma in Japanese patients. *J Surg Oncol* 108: 557-562, 2013.
22. Sobin LH, Gospodarowicz MK and Wittekind CH: International Union Against Cancer: TNM Classification of Malignant Tumors. 7th edition. Wiley-Blackwell, New York, 2009.
23. Oya H, Kanda M, Takami H, *et al*: Overexpression of melanoma-associated antigen D4 is an independent prognostic factor in squamous cell carcinoma of the esophagus. *Dis Esophagus*: Oct 2, 2013 (Epub ahead of print). doi: 10.1111/dote.12156.
24. Kanda M, Shimizu D, Nomoto S, *et al*: Prognostic impact of expression and methylation status of DENN/MADD domain-containing protein 2D in gastric cancer. *Gastric Cancer*: Apr 3, 2014 (Epub ahead of print).
25. Shimizu D, Kanda M, Nomoto S, *et al*: Identification of intragenic methylation in the TUSC1 gene as a novel prognostic marker of hepatocellular carcinoma. *Oncol Rep* 31: 1305-1313, 2014.
26. Takai D and Jones PA: The CpG island searcher: a new WWW resource. *In Silico Biol* 3: 235-240, 2003.
27. Hibino S, Kanda M, Oya H, *et al*: Reduced expression of DENND2D through promoter hypermethylation is an adverse prognostic factor in squamous cell carcinoma of the esophagus. *Oncol Rep* 31: 693-700, 2014.
28. Saandi T, Baraille F, Derbal-Wolfrom L, *et al*: Regulation of the tumor suppressor homeogene Cdx2 by HNF4alpha in intestinal cancer. *Oncogene* 32: 3782-3788, 2013.
29. Kanda M, Shimizu D, Nomoto S, *et al*: Clinical significance of expression and epigenetic profiling of TUSC1 in gastric cancer. *J Surg Oncol* 110: 136-144, 2014.
30. Nagai H, Pineau P, Tiollais P, Buendia MA and Dejean A: Comprehensive allelotyping of human hepatocellular carcinoma. *Oncogene* 14: 2927-2933, 1997.
31. Li SP, Wang HY, Li JQ, *et al*: Genome-wide analyses on loss of heterozygosity in hepatocellular carcinoma in Southern China. *J Hepatol* 34: 840-849, 2001.
32. Walesky C, Edwards G, Borude P, *et al*: Hepatocyte nuclear factor 4 alpha deletion promotes diethylnitrosamine-induced hepatocellular carcinoma in rodents. *Hepatology* 57: 2480-2490, 2013.
33. Ehehalt F, Rummele P, Kersting S, *et al*: Hepatocyte nuclear factor (HNF) 4alpha expression distinguishes ampullary cancer subtypes and prognosis after resection. *Ann Surg* 254: 302-310, 2011.