

# Overexpression of CDC25A Phosphatase Is Associated with Hypergrowth Activity and Poor Prognosis of Human Hepatocellular Carcinomas<sup>1</sup>

Xundi Xu, Hirofumi Yamamoto,<sup>2</sup> Masato Sakon, Masayoshi Yasui, Chew Yee Ngan, Hiroki Fukunaga, Tetsushi Morita, Minoru Ogawa, Hiroaki Nagano, Shoji Nakamori, Mitsugu Sekimoto, Nariaki Matsuura, and Morito Monden

Department of Surgery and Clinical Oncology, Graduate School of Medicine [X. X., H. Y., M. S., M. Y., C. Y. N., H. F., T. M., M. O., H. N., S. N., M. S., M. M.] and Department of Pathology, School of Allied Health Science, Faculty of Medicine [N. M.], Osaka University, Osaka 565-0871, Japan

## ABSTRACT

**Purpose and Experimental Design:** *CDC25* genes are cell cycle-activating phosphatases that positively regulate the activity of cyclin-dependent kinase. *CDC25A* and *CDC25B*, being oncogenes, are overexpressed in a variety of human malignancies. To investigate the potential roles of *CDC25s* in hepatocellular carcinoma (HCC), expression of *CDC25A* and *CDC25B* was examined in human HCC samples.

**Results:** Reverse transcription-PCR showed that overexpression of *CDC25A* and *CDC25B* mRNAs was found in 9 of 13 (69%) and 4 of 13 (31%) HCCs, respectively. Immunohistochemistry of 59 HCCs showed marked increase in *CDC25A* expression, but not *CDC25B*, in HCC compared with noncancer tissues, and high expression of *CDC25A* in 33 of 59 (56%) HCCs. Overexpression of *CDC25A* in HCC was confirmed by Western blot analysis. High expression of *CDC25A* was associated with dedifferentiated phenotype and portal vein invasion ( $P = 0.001$  and  $0.031$ , respectively), and expression of *CDC25A* correlated well with proliferating cell nuclear antigen labeling index ( $P = 0.005$ ). Univariate analysis indicated that high expression of *CDC25A* and proliferating cell nuclear antigen were both significant predictive factors for shorter disease-free survival ( $P = 0.004$

and  $0.039$ , respectively). Multivariate analysis indicated that *CDC25A* was an independent prognostic marker for disease-free survival (risk ratio for cancer relapse,  $2.98$ ;  $P = 0.029$ ), even when analyzed with several clinicopathologic factors. On the other hand, expression of *CDC25B* did not correlate with any clinicopathological features.

**Conclusion:** Our findings suggest that *CDC25A*, but not *CDC25B*, could be used as an independent prognostic marker for HCC. Our data would also contribute to forward understanding of tumor biology of HCC that is associated with cell cycle regulation.

## INTRODUCTION

Primary HCC<sup>3</sup> is one of the most common tumors in Southeast Asia and Africa with an estimated incidence of ~30/100,000 men per year. The prognosis of HCC is generally poor, and the 5-year survival rate is limited to 25–49% after surgery (1, 2). In an effort to understand the biological features of this aggressive type of carcinoma, several studies have shown alterations of several key molecules including  $\beta$ -catenin, Rb, p53, and c-myc (3–5), but much remains to be clarified.

Mammalian cell cycle is controlled by sequential activation and inactivation of a family of CDKs. CDKs interact with a specific subset of cyclins during different phases of the cell cycle to determine the proper timing and coordination of cell cycle progression (6). The negative control of CDK is exerted by various CDK inhibitors (7). An alternative negative regulation of CDK action is modulated by phosphorylation of threonine 14 and tyrosine 15 residues by Wee-1 or Mik1 (8). On the other hand, positive regulation of CDK activity is exerted by CDK activating kinase or cell cycle-activating phosphatase *CDC25* genes. The latter molecules activate CDK by removal of the inhibitory phosphates of threonine and tyrosine residues in the ATP-binding sites of the CDK. Three *CDC25*-related genes, *CDC25A*, *CDC25B*, and *CDC25C*, which share approximately 40–50% amino acid identity, have been identified in humans (9–11). *CDC25* phosphatases act at different points of the cell cycle, including G<sub>1</sub>-S and G<sub>2</sub>-M transition (9, 10, 12).

Dysregulation of cell cycle progression has been characterized in a variety of human malignancies (13). HCC tissues overexpress cyclins D1 and E (14, 15). The CDK inhibitor p21waf1/cip1 is reduced, methylation of the *p16INK4* gene occurs in the promoter region, and p27Kip1 appears to be

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<sup>2</sup> To whom requests for reprints should be addressed, at Department of Surgery and Clinical Oncology, Graduate School of Medicine, Osaka University, 2-2 Yamada-oka, Suita City, Osaka 565-0871, Japan. Phone: 81-6-6879-3251; Fax: 81-6-6879-3259; E-mail: kobunyam@surg2.med.osaka-u.ac.jp.

<sup>3</sup> The abbreviations used are: HCC, hepatocellular carcinoma; CDK, cyclin-dependent kinase; PCNA, proliferating cell nuclear antigen; RT-PCR, reverse transcription-PCR; PBGD, porphobilinogen deaminase; LI, labeling index; CH, chronic hepatitis;  $\beta$ -gal,  $\beta$ -galactosidase; T/N, tumor:nontumor; DFS, disease-free survival.

decreased in a subset of HCCs (15–17). Recent studies point out the relevance of CDC25 phosphatases in human neoplasms. In the CDC25 family, A and B types, but not CDC25C, appear to be potential oncogenes as they have been found to transform primary murine fibroblasts in cooperation with either mutated *Ha-ras* or loss of *Rb1* (18). Transgenic mice that overexpress the *CDC25B* gene display enhanced sensitivity to carcinogen 9,10-dimethyl-1,2-benzanthracene (19) or develop mammary gland hyperplasia (20). Concordant to *in vitro* and *in vivo* findings, overexpression of CDC25A and CDC25B has been demonstrated in many types of human malignancies (21–28).

Although dysfunction of the cell cycle machinery occurs infrequently in human HCC, the expression and clinical significance of CDC25A and CDC25B proteins have not yet been elucidated. Therefore, in the present study, we examined 59 primary HCCs immunohistochemically and determined the correlation between the levels of these proteins, and various clinical and pathological features including prognosis. In addition, in an effort to assess proliferation of HCC cells, we examined the expression of a nonhistone nuclear protein, PCNA, which accumulates from late G<sub>1</sub> to S-phase and acts as an adjunct to DNA polymerase (29).

## MATERIALS AND METHODS

**Cell Culture.** HEK 293 cells were obtained from the American Type Culture Collection (ATCC CRL-1573) and cultured in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin in 5% CO<sub>2</sub> at 37°C.

**Patients and Tissue Specimens.** Tissue samples were obtained from 59 patients with HCC (49 males and 10 females; 44–76 years of age; mean, 61 ± 6 years), who underwent hepatectomy at the Department of Surgery and Clinical Oncology, Osaka University. Before hepatectomy for HCC, 31 patients were treated with transarterial embolization. All of the patients had hepatitis C virus infection but none had hepatitis B viral infection. The mean follow-up period in the prognosis study was 3.4 ± 1.8 years. The resected surgical specimens were fixed in 10% neutral buffered formalin, processed through graded ethanol solution, and embedded in paraffin. A piece of each tissue sample was immediately frozen in liquid nitrogen and stored at –80°C for analysis by RT-PCR and Western blotting.

**Pathological Examination.** Tissue sections (4-µm thick) were deparaffinized in xylene, rehydrated with graded concentrations of ethanol, and stained with H&E solution. Pathological diagnosis of tissues into nontumor and tumor tissues was determined by a pathologist (N. M.) who was blinded to the clinical background. For 59 nontumor tissues, the presence of active inflammation and cirrhotic nodule was examined and 23 cases with CH and 36 with liver cirrhosis were identified. Tumor tissues were examined for the following characteristics: tumor site, cell differentiation (well, moderately, poorly differentiated, or undifferentiated), hepatic vein invasion, portal vein invasion, number of tumors, septal formation, capsular formation, and capsular invasion.

**Antibodies.** The rabbit polyclonal antibodies for CDC25A and CDC25B phosphatases and their blocking pep-

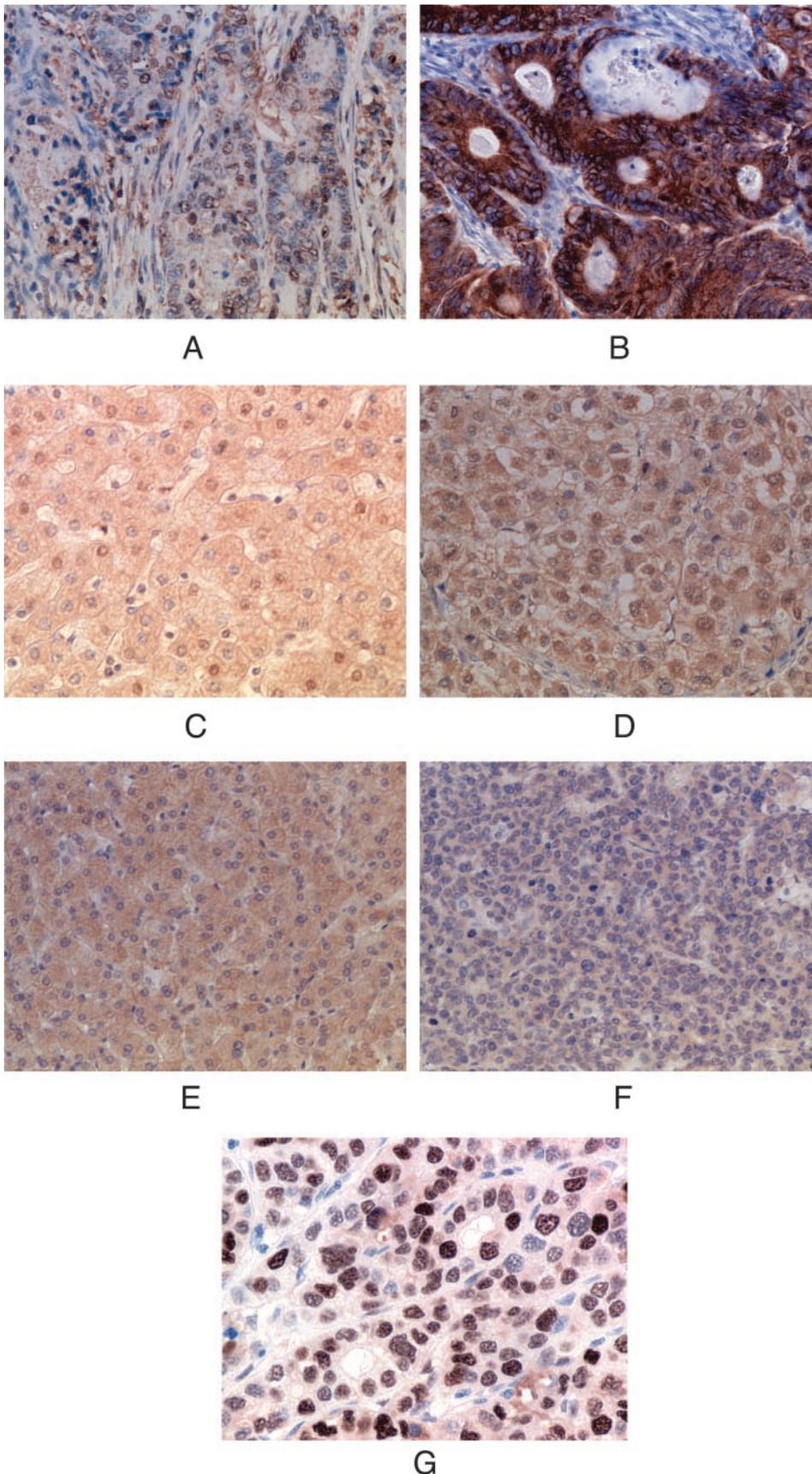
tides that were used as immunogen for generation of the antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA; Refs. 25, 26). The mouse monoclonal antihuman PCNA antibody was purchased from DAKO (Carpinteria, CA; Ref. 29).

**Immunohistochemistry.** Immunostaining was performed on the TechMate Horizon automated staining system (DAKO, Glostrup, Denmark) using the Vectastain ABC-peroxidase kit (Vector Laboratories, Burlingame, CA), as described previously (25, 26). The primary antibodies were applied to the sections at the following dilutions: anti-CDC25A antibody, 1:100; anti-CDC25B antibody, 1:50; and anti-PCNA antibody, 1:400. Counter nuclear staining was performed briefly with hematoxylin solution. For the positive controls of CDC25 phosphatase, sections of colon cancer expressing intense CDC25A or CDC25B protein (25) were included in each staining procedure. For negative controls, nonimmunized rabbit or mouse IgG (Vector Labs), or PBS was used as a substitute for the primary antibody. In addition, absorption tests were performed to verify the specificity of the CDC25A and CDC25B antibodies.

**Immunohistochemical Evaluation.** All of the immunostained sections were evaluated in a blinded manner without knowledge of the clinical and pathological parameters of the patients. For assessment of CDC25A and PCNA, four high-power fields in each specimen were selected randomly, and nuclear staining was examined under high power magnification. More than 500 cells were counted to determine the LI, which represented the percentage of immunostained cells relative to the total number of cells. Because CDC25B expression was found mainly in the cytoplasm and its level was generally less than strong, the intensity of staining was scored on the two scales where “positive” represented moderate staining and “negative” indicated none or only faint staining. In half of the samples, staining was repeated twice to avoid possible technical errors, but similar results were obtained in these samples. The above procedures of evaluation were performed by X. X. The obtained results were confirmed by two investigators (H. Y. and N. M.) using a multihead microscope, and a consensus was achieved.

**Semiquantitative RT-PCR Analysis for CDC25A and CDC25B mRNAs.** Semiquantitative analysis for expression of CDC25B or CDC25A mRNA was performed by the multiplex RT-PCR technique, using PBGD as the internal standard, as described previously (25, 26). PCRs were performed in a total volume of 25 µl reaction mixture containing 1 µl of cDNA template, 1× Perkin Elmer PCR buffer, 1.5 mM MgCl<sub>2</sub>, 0.8 mM deoxynucleotide triphosphates, 20 pmol of each primer for CDC25A or CDC25B, 4 pmol each for PBGD, and 1 unit of TaqDNA Polymerase (AmpliTaq Gold; Roche Molecular Systems, Inc., Belleville, NJ). The sequences of these PCR primers and PBGD were described previously (25, 26, 30). The sizes of the amplicons for CDC25A, CDC25B, and PBGD were 272, 416, and 127 bp, respectively.

**Western Blot Analysis.** Western blotting was performed as described previously (25). Briefly, 50 µg of the total protein were subjected to 10% PAGE, followed by electroblotting onto a polyvinylidene difluoride membrane. After blocking in 5% skim milk, the membrane was incubated with 1 µg/ml CDC25A antibody, followed by incubation with the secondary antibody at



*Fig. 1* Immunostaining with antihuman CDC25A and CDC25B antibodies in colorectal carcinoma tissues. *A*, a representative colorectal carcinoma tissue with low expression level of CDC25A. *B*, a representative colorectal carcinoma with high expression level of CDC25B. Immunostaining with antihuman CDC25A antibody in noncancerous tissue of the liver (*C*) and HCC tissue (*D*). *C*, note the low expression level of CDC25A in noncancerous tissue of the liver. *D*, a representative HCC expressing a high level of CDC25A (LI = 42%). Immunostaining with antihuman CDC25B antibody in normal and noncancerous tissue of the liver (*E*) and HCC tissue (*F*). *E*, moderate expression of CDC25B was noted in the noncancerous tissue. *F*, weak expression of CDC25B was noted in the cytoplasm of hepatoma cells. Immunostaining with antihuman PCNA antibody in HCC tissue (*G*). *G*, a representative HCC that expressed a high level of PCNA (LI = 49%). Magnification: *A–F*,  $\times 100$ .

a dilution of 1:3000. For detection of the immunocomplex, the enhanced chemiluminescence Western blot detection system (Amersham, Aylesbury, United Kingdom) was used.

**Transfection and  $\beta$ -Gal Staining** The human CDC25A cDNA (length 1.6 Kb) was obtained from D. Beach (Wolfson Institute for Biomedical Research, University College, London) (18) and subcloned into the *NotI* site of the pcDNA3 vector (Invitrogen, Carlsbad, CA) in the sense orientation. HEK 293 cells were seeded 24 h before transfection at a density of  $1 \times 10^5$ /well in 6-cm dishes. To monitor the transfection efficiency, either the CDC25A plasmid or pcDNA3 vector was cotransfected with a cytomegalovirus  $\beta$ -gal plasmid at a ratio of 2:1 (1  $\mu$ g:0.5  $\mu$ g), using the lipofectin reagent (Life Technologies, Inc., Gaithersburg, MD), as recommended by the supplier. Forty-eight h after transfection the cells were collected. For immunostaining, cells were fixed on the glass slides in 10% buffered formalin for 10 min and in 70% ethanol solution for 30 min. For  $\beta$ -gal staining,  $\beta$ -gal staining kit (ACTIVE MOTIF, Carlsbad, CA) was used. Briefly, cells were fixed with 0.7% formaldehyde and 0.05% glutaraldehyde solution, and washed twice with PBS.

$\beta$ -Gal-introduced cells were visualized by incubation with  $\beta$ -gal staining solution (4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM  $MgCl_2$ , and 0.02% 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside) at 37°C for 60 min.

**Statistical Analysis.** Statistical analysis was performed using the Statview J-5.0 program (Abacus Concepts, Inc., Berkeley, CA). The postoperative period extended from the date of surgery to the date of the last follow-up or death. Survival was censored if the patient was still alive or died from other causes. The Kaplan-Meier method was used to estimate recurrence or death from HCC, and the log-rank test was used to examine statistical significance. A Cox proportional hazards model was used to assess the risk ratio under simultaneous contribution from several covariates. The associations between the discrete variables were assessed using the  $\chi^2$  test or Fisher's exact probability test. Mean values were compared using the Mann-Whitney test. Data are expressed as mean  $\pm$  SD. A  $P < 0.05$  denoted the presence of a statistically significant difference.

## RESULTS

**Specificity of CDC25A and CDC25B Antibodies.** Immunohistochemical assays were performed on a series of 59 paired HCCs and their matched nontumor tissues. Immunohistochemical staining of sections of colon cancer expressing CDC25A and CDC25B served as positive controls (Fig. 1, A and B; Ref. 25). In contrast, no staining was observed when the primary antibody was substituted with nonimmunized rabbit IgG or PBS (data not shown). The use of antibody preabsorbed with excess amount of immunogens abolished staining (data not shown), indicating that these antibodies were highly specific to CDC25A and CDC25B proteins on tissue sections. The CDC25A protein was mainly found in the nucleus, whereas the CDC25B protein was localized in the cytoplasm, consistent with the results of previous studies showing CDC25A expression in the nuclear fraction and abundant CDC25B expression in the cytoplasm (31, 32).

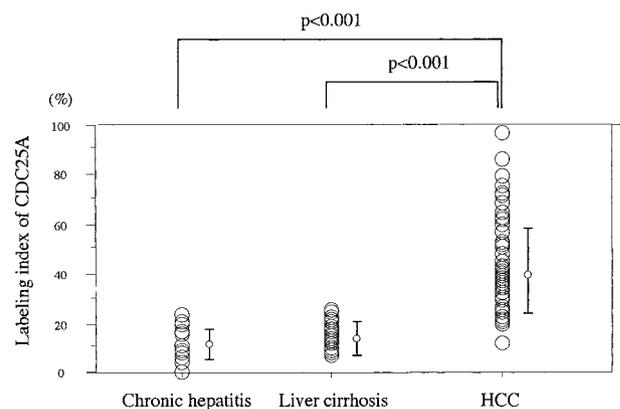


Fig. 2 LI of CDC25A in CH, liver cirrhosis, and HCC. HCC displays significantly higher LI than CH or liver cirrhosis (both for  $P < 0.001$ ); bars,  $\pm$ SD.

**Immunohistochemistry for CDC25A.** CDC25A protein was expressed in all of the nontumor and carcinoma tissues tested, mainly in the nucleus. The percentage of cells positive for nuclear CDC25A was relatively low in noncancerous tissues (Fig. 1C); the LI was  $13.5 \pm 4.9\%$  in CH-based hepatocytes ( $n = 23$ ) and  $15.7 \pm 4.3\%$  in cirrhotic livers ( $n = 36$ ). In carcinoma tissues, 25 cases (42.4%) expressed nuclear CDC25A with moderate to strong intensity (Fig. 1D), whereas the remaining 34 cases (57.6%) displayed a weak CDC25A expression. LI varied widely in carcinoma tissues with a mean value of  $42.3 \pm 12.7\%$ . The difference in LI for CDC25A between HCC and CH-based hepatocytes or cirrhotic livers was significant ( $P < 0.001$  for both; Fig. 2), and overexpression rate of the CDC25A protein was 78% (46 of 59).

**Transfection Assay and Western Blot Analysis.** The above immunohistochemical assay showed that most of the HCCs expressed increased levels of CDC25A protein when compared with noncancerous tissues. To additionally rule out a possibility of nonspecific staining with the CDC25A antibody, we performed additional experiments for confirmation of the results. Transfection assay in duplicate cultures showed that transfection efficiency using  $\beta$ -gal construct was  $\sim 12\%$  in pcDNA3-transfected cultures and 10% in CDC25A-transfected cultures, respectively (Fig. 3, A and B). By staining with the CDC25A antibody, pcDNA3-transfected cultures did not express nuclear CDC25A, whereas CDC25A-transfected cultures did display an intense nuclear CDC25A staining in  $\sim 5\%$  of the cells (Fig. 3, C and D). The findings indicate that the CDC25A antibody specifically reacts with CDC25A protein. Western blot analysis for CDC25A in six pairs of cancer and its noncancer counterpart revealed that majority of HCCs indeed exhibited overexpression, and a subset of HCCs expressed considerable levels of CDC25A (Fig. 4). There was a significant correlation between CDC25A level on immunoblot and LI determined by immunohistochemistry ( $\gamma^2 = 0.672$ ;  $P < 0.001$ ).

**Immunohistochemistry for CDC25B.** Approximately half of the nontumor tissues (28 of 59: 47.5%; 11 of 23 CH and 17 of 36 cirrhosis) expressed the CDC25B protein in moderate intensity (Fig. 1E) and the other half (31 of 59: 52.5%) dis-

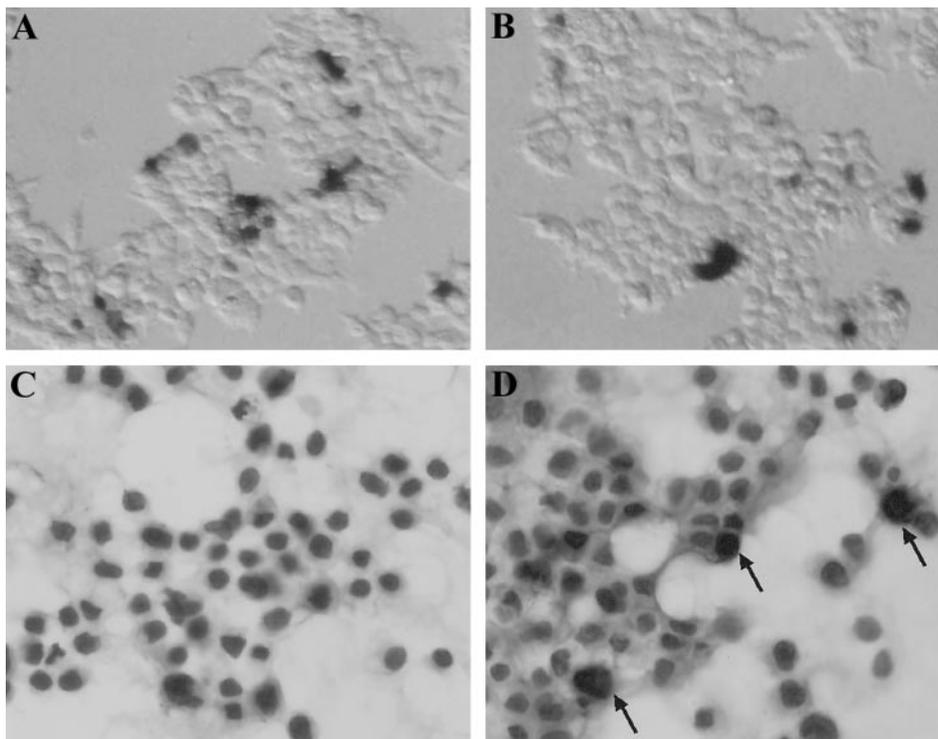


Fig. 3 Transfection assays. Transfection efficiency was ~12% in pcDNA3-transfected cultures (A) and 10% in CDC25A-transfected cultures (B).  $\beta$ -Gal-positive HEK 293 cells were in blue. pcDNA3 transfected cultures did not express nuclear CDC25A (C), whereas CDC25A-transfected cultures did display an intense nuclear CDC25A staining in ~5% cells (D). The arrows indicate positive staining.

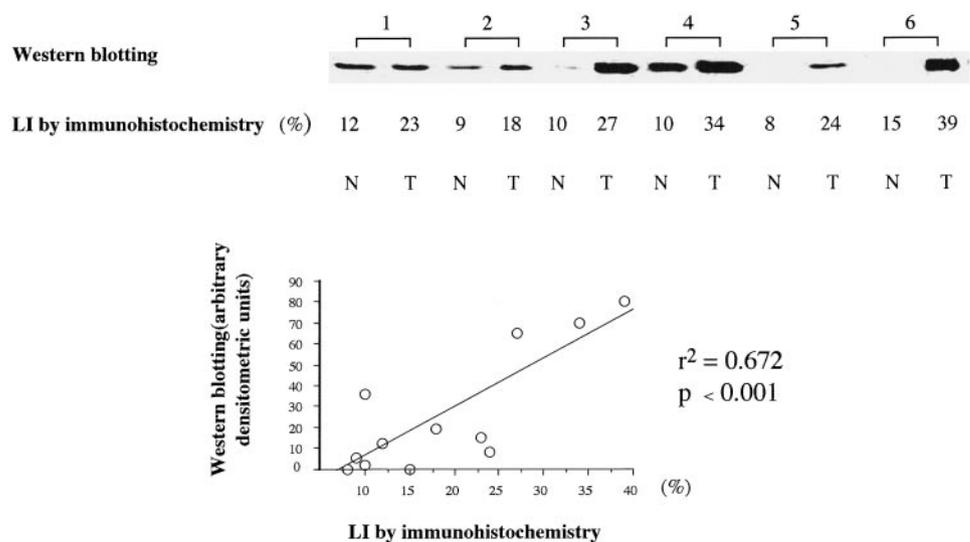


Fig. 4 Western blot analysis for CDC25A. Western blotting was performed on six pairs of HCC and its noncancer counterpart. There was a significant correlation between CDC25A level on immunoblot and LI determined by immunohistochemistry ( $\gamma^2 = 0.672$ ;  $P < 0.001$ ).

played none or only faint CDC25B expression. On the other hand, over half of the HCCs expressed the CDC25B protein with none or only faint intensity (39 of 59: 66.1%; Fig. 1F) and the remaining 20 HCCs (33.9%) expressed it with moderate intensity. Among the HCC samples, none expressed as strong a CDC25B expression as the positive control sample of colon cancer shown in Fig. 1B. As a result, 12 of 59 HCCs (20.3%) expressed increased levels of the CDC25B protein, compared with their noncancer counterparts.

**RT-PCR Analysis for CDC25A and CDC25B mRNAs.**

RT-PCR analysis was performed using 13 paired nontumor and tumor mRNA extracts. The relative value of CDC25A and CDC25B band to PBGD band was calculated, and the T:N ratio was determined in each case. Three representative cases are shown in Fig. 5. The T:N ratios of cases 1, 2, and 3 were 2.5, 3.1, and 2.2 for CDC25A, and 1.3, 1.6, and 0.4 for CDC25B, respectively. When the T:N ratio of >2.0 was defined as over-expression, CDC25A was overexpressed in 9 of 13 (69%) cases

Fig. 5 RT-PCR analysis for CDC25A and CDC25B mRNAs. RT-PCR analysis was performed for CDC25A (top panel) or CDC25B (bottom panel) mRNA in paired normal and carcinoma tissues. The value of CDC25A or CDC25B band relative to PBGD band was calculated for each sample, and the T:N ratio was determined in each case.

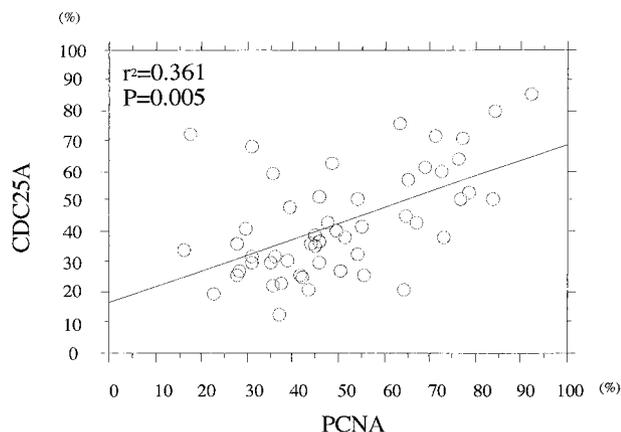
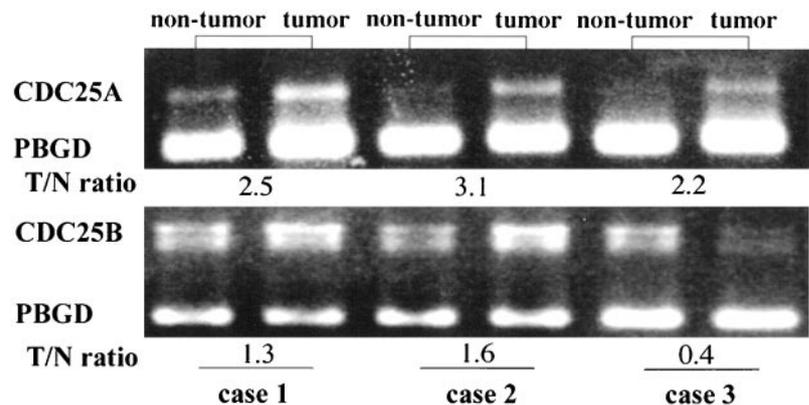


Fig. 6 Relationship between CDC25A and PCNA expression, significant correlation was found ( $\gamma^2 = 0.361$ ;  $P < 0.01$ ).

tested, whereas CDC25B was overexpressed in 4 of 13 (31%) cases. When mRNA levels of CDC25A or CDC25B were compared with expression levels detected by immunohistochemistry, no discrepant results were found (data not shown).

**PCNA Expression.** To examine the possible involvement of CDC25 phosphatases in growth of carcinoma cells, we examined PCNA expression and compared it with that of CDC25A and CDC25B. Carcinoma tissues displayed a wide variety of PCNA expression ranging from 16 to 92%, with a mean value of  $49.4 \pm 14.8\%$ , which was significantly higher than  $20.5 \pm 7.2\%$  in CH-based hepatocytes, and  $15.5 \pm 6.0\%$  in cirrhotic hepatocytes ( $P < 0.001$  for both; Fig. 1G). When the patients were divided into two groups according to a cutoff of the mean value of PCNA expression, high PCNA expression was associated with dedifferentiated carcinoma ( $P = 0.012$ ) but not with other clinicopathological parameters (data not shown). Moreover, the expression of CDC25A and PCNA displayed a clear correlation (Fig. 6;  $P = 0.005$ ;  $\gamma^2 = 0.361$ ), whereas no correlation was found between CDC25B expression and PCNA expression (data not shown).

**Relationship between Clinicopathological Parameters and CDC25A or CDC25B Expression.** For statistical analysis of the expression of CDC25A, the carcinoma specimens

Table 1 Relationship between CDC25A expression and clinicopathological parameters in cases with HCC

		Number of cases	CDC25A		P
			Low	High	
Age	$\geq 60$	49	23	26	N.S. <sup>a</sup>
	$< 60$	10	3	7	
Gender	Male	49	23	26	N.S.
	Female	10	3	7	
Tumor size	$< 2$ cm	18	5	13	N.S.
	$\geq 2$ cm	41	21	20	
Histological grade <sup>b</sup>	Well/mod.	38	23	15	0.001 <sup>c</sup>
	Poor./undiff.	21	3	18	
Hepatic vein invasion	Yes	3	0	3	N.S.
	No	56	26	30	
Portal vein invasion	Yes	9	1	8	0.031 <sup>c</sup>
	No	50	25	25	
Number of tumor <sup>d</sup>	Solitary	42	20	22	N.S.
	Multiple	17	6	11	
Septal formation	Yes	41	19	22	N.S.
	No	18	7	11	
Capsular formation	Yes	46	22	24	N.S.
	No	13	4	9	
Capsular invasion <sup>e</sup>	Yes	21	7	14	N.S.
	No	25	15	10	

<sup>a</sup> N.S., not significant.

<sup>b</sup> Mod., moderately; poor., poorly; undiff., undifferentiated.

<sup>c</sup> Statistically significant.

<sup>d</sup> This category includes both intrahepatic metastasis and tumors generated by multicentric carcinogenesis.

<sup>e</sup> The capsular invasion is composed in the category of capsular formation.

were divided into two groups: 33 (56.0%) high expressors and 26 (44.0%) low expressors, according to the percentage of nuclear CDC25A-positive cells, using a cutoff level of 42.3%, representing the mean value of CDC25A expression. We then analyzed the relationship between CDC25A expression and various clinicopathological parameters listed in Table 1. There was a significant correlation between high expression of CDC25A and dedifferentiated phenotype (poorly differentiated carcinoma and undifferentiated carcinoma), and portal vein invasion ( $P = 0.001$  and  $0.031$ , respectively). No correlation was found between CDC25A expression and other variables including age, gender, tumor size, hepatic vein invasion, number

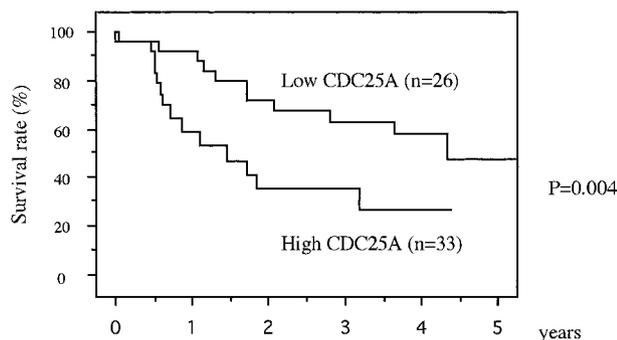


Fig. 7 Kaplan-Meier curves for DFS according to CDC25A expression in 59 patients with HCC. High expression of CDC25A was significantly associated with worse prognosis ( $P = 0.004$ ).

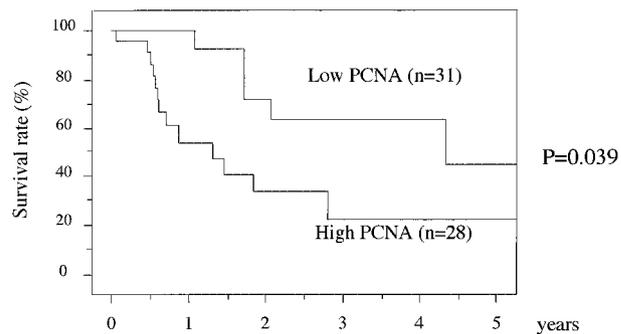


Fig. 8 Kaplan-Meier curves for DFS according to PCNA expression in 59 patients with HCC. High expression of PCNA was significantly associated with worse prognosis ( $P = 0.039$ ).

of tumor, septal formation, capsular formation, and capsular invasion.

With regard to CDC25B expression, comparison between the moderate expression group ( $n = 20$ ) and none or faint expression group ( $n = 39$ ) showed no significant differences in the correlations between the expression and the clinicopathological features listed in Table 1 (data not shown).

**Analysis of Survival Rates.** We then analyzed the DFS rates based on CDC25A and CDC25B expression, as well as clinicopathological features of HCCs. Univariate analysis showed that high expression of CDC25A was a significant predictor of disease relapse, when assessed by Kaplan-Meier curves (Fig. 7;  $P < 0.01$ ). Among the various parameters listed in Table 1, the histological grade and portal vein invasion were also indicative of disease relapse ( $P = 0.003$  and  $P = 0.002$ , respectively). Hepatic vein invasion showed a significant value, but the positive cases were too small (only 3 of 59) for adequate statistical analysis. We also found that PCNA expression was also indicative of disease relapse (Fig. 8;  $P = 0.039$ ). In contrast, expression of CDC25B and other clinicopathological parameters were not significant prognostic factors.

Multivariate analysis using the Cox regression model showed that CDC25A expression was a significant covariate ( $P = 0.029$ ; Table 2), as well as portal vein invasion ( $P = 0.017$ ) and PCNA expression ( $P = 0.022$ ). The relative risk of cancer relapse in patients with carcinomas expressing high levels of CDC25A was 2.98 that of patients with tumors expressing low levels of CDC25A.

## DISCUSSION

CDC25A and CDC25B are overexpressed in various types of human carcinomas (21–28). Importantly, overexpression of CDC25A and CDC25B is often associated with malignant features of human neoplasms, which include poor prognosis of carcinomas of the esophagus, breast, colon, and ovary, advanced stage gastric cancer, poorly differentiated non-small cell lung cancer, and aggressive types of lymphoma (22–28). These findings suggest that CDC25 phosphatases are useful markers for the malignant potential of various carcinomas, and they may function to enhance cancer growth and expansion, although information regarding their biological functions in malignant cells is limited.

Our results showed a preferential expression of CDC25A over CDC25B in HCC. The overexpression rate for CDC25A was 69% and 78%, at RNA level and protein level, respectively, whereas those for CDC25B were only 31% and 20%. These findings are in contrast with our previous reports on concurrent overexpression of CDC25A and CDC25B in carcinomas of the colon and esophagus (25, 26). The mechanism for CDC25A overexpression in HCC is unknown at present, although a few molecules such as c-myc, E2F, transforming growth factor  $\beta$  could be considered as a key candidates to affect the synthesis of CDC25A (33–35). Our data indicated that CDC25A was not only up-regulated but also associated with aggressive cancer phenotypes including portal vein invasion and dedifferentiated histology (Table 1). Moreover, univariate and multivariate analyses showed that high expression of CDC25A was associated significantly with disease relapse of HCC. In contrast, CDC25B expression was not associated with conventional clinicopathological parameters, and it was not helpful in predicting prognosis. These data again highlight the relevance of CDC25A in HCC rather than CDC25B. Then what is the functional significance of CDC25A molecule in HCC?

One of the mechanisms related to malignant potential could be that CDC25A may function to inhibit cellular apoptosis. The survival promoting activity by CDC25A was demonstrated in the serum-starved condition (36). It is postulated that increased expression of CDC25A may contribute to reduced cellular responsiveness to oxidative stress (37). However, at present, only limited evidence is provided in relation to apoptosis. On the other hand, there is cumulative evidence concerning its contribution to cell cycle control. Previous studies have shown that CDC25A is a rate-limiting, positive regulator of the cell cycle. A gain in CDC25A activity can result in defects in transforming growth factor  $\beta$  antiproliferative responsiveness (35). Ablation of CDC25A function by microinjection of a specific antibody blocks entry into S phase (12). Conversely, inducible overexpression of CDC25A leading to activation of cyclin E-Cdk2 and cyclin A-Cdk2 revealed that these complexes act as critical targets for CDC25A (38, 39). This evidence emphasizes the relevance of CDC25A in  $G_1$ -S transition and, accordingly, it is suggested that disturbance of  $G_1$ -S transition may be important in the progression of human HCC.

Human HCC takes a unique natural course of tumor pro-

Table 2 Multivariate analysis (Cox proportional hazards model)

Categories <sup>a</sup>	P	Risk ratio	95% Confidence interval
CDC25A (H:L)	0.029	2.98	0.126–0.894
Portal vein invasion (yes:no)	0.017	4.13	0.075–0.774
PCNA (H:L)	0.022	2.66	1.154–6.107
Differentiation (well/mod.:poor./undiff.)	0.541	1.31	0.319–1.822
Number of tumor (solitary:multiple)	0.406	1.49	0.580–3.844
Capsular formation (yes:no)	0.133	2.16	1.170–1.263

<sup>a</sup> H:L, high:low; well/mod.:poor./undiff., well/moderately differentiated:poorly differentiated/undifferentiated.

gression. Early HCC usually shows well-differentiated phenotype and grows relatively slowly. As the tumor stage advances, HCC gradually grows faster, associated with dedifferentiation (40, 41). In accordance with this model, we found that high expression of PCNA was significantly associated with poorly differentiated tumors. Furthermore, PCNA expression was associated significantly with the disease relapse, consistent with reports from other laboratories (42, 43). Likewise, active cell growth is an important aspect that defines the prognosis of patients with carcinomas of the breast and lung (44, 45), whereas cell proliferation alone does not always result in fatal events in certain types of human malignancies, including carcinomas of the colon (46, 47). Probably other features apart from cell growth, such as invasive capability and metastasis potential, are more important in this cancer type. Therefore, in the case of HCC, it is postulated that uncontrolled cell growth may particularly play a crucial role in disease progression. Finally, we found a clear correlation between PCNA index and CDC25A expression. Taken together, the present work suggests the following scenario. Increased level of CDC25A in HCC facilitates progression of the cell cycle from G<sub>1</sub> phase to S phase, leading to active DNA synthesis. Cancer cells then acquire a high proliferative activity and cause disease recurrence.

In conclusion, we have demonstrated in the present study that CDC25A, but not CDC25B, is a novel independent prognostic marker for patients with HCC. Our findings also suggest that CDC25A might be a therapeutic target for an aggressive form of HCC by using a CDC25A-specific inhibitor or antisense technology. Indeed, several inhibitors are being developed, including novel arylating K vitamin analogues, vitamin D3 analogues, and steroidal derived inhibitors, and some of them showed antiproliferative activity in cancer cell lines including HCC (48, 49).

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