表 1 HCCに対する動注化学療法の主な成績

薬剤	報告者（年）	症例数	奏効率	備考
単剤				
DDP-H（動注用CDDP）	後期II相試験	80	34%	
CDDP	Carr（2002）	26	42%	
EPI	永末（1986）	53	15%	
SMANCS	田口（1991）	167	36%	
多剤併用				
5-FU/CDDP	永松（2001）	45	53%	
5-FU/CDDP	Ando（2002）	48	48%	Vp 2～4
5-FU/CDDP/ADM	太田（2003）	9	44%	Vp 2～4
5-FU/IFN	Sakon（2000）	8	63%	Vp 3～4
5-FU/IFN	永野（2003）	40	48%	Vp 3～4

ADM: doxorubicin, EPI: epirubicin, CDDP: cisplatin

感受性で，肝硬変合併による肝障害の問題も無視できず，少なくとも全身化学療法の果たす役割は低いと考えられる。その反面，HCCは進行期でも比較的肝内に病変がとどまることから，高い局所濃度が得られる肝動注による予後改善に期待がもたれ，種々のレジメが模索されているが，投与量や投与方法について一定の見解はない。本稿では，本邦において最近注目されている動注薬剤やレジメの動向について述べる。

I．投薬方法

進行期に多い中・低分化型HCCは，通常肝動脈に栄養される多血性腫瘍であり，動注により病変への高い薬剤濃度分布が図れる。投薬方法は大別して，one-shot動注および皮下埋込式リザーバーによる持続動注がある。one-shot動注は，血管造影手技により抗癌剤の単回投与の反復を行うもので，その都度入院を繰り返す必要がある。一方，リザーバーによる持続動注では，外来でも簡便に抗癌剤の持続・反復投与が行える。IVRによる経皮的留置技術やポート・カテーテルなどの留置器具の進歩は目覚しく，良好な肝内薬剤分布や血管開存性の向上に寄与している。留置方法は大腿動脈あるいは左鎖骨下動脈経由で，側孔付カテーテルを肝動脈末梢（肝末梢固定法）や胃十二指腸動脈（GDAコイル法）に先端固定留置し，消化管・膵保護のため適宜コイル塞栓により血流改変を加える方法が一般化しており，カテーテル逸脱や閉塞などのトラブルも減少した[2]。

II．動注における薬剤選択

One-shot動注では濃度依存性の高いepirubicin, MMCやCDDPなどが適している。最近，肝動注用CDDP製剤が新たに保険認可されたが，その詳細は後述する。一方，リザーバー動注では時間依存性の高い5-FUをkey drugとしてcisplatin（CDDP），leucovorin，インターフェロン（interferon: IFN）などをbiochemical modulatorとして用いる傾向が強い。腫瘍への選択性や薬剤濃度維持など効果増強を図るため，抗癌剤担体としての油性造影剤Lipiodolや，一時的塞栓物質である微小澱粉球（スフェレックス）を併用する方法もある。しかし，同時に抗癌剤の肝内停滞により非癌部肝組織への障害も高まるため，併用の可否は肝予備能に依存する。また，いずれも塞栓効果を有するためX線透視下でオーバーフローしないことを確認しながらの注入が原則である。特にリザーバーからのLipiodolの使用は，カテーテル材質の合成樹脂の破損の原因となるので注意が必要である。Lipiodolとの懸濁を前提とした油溶性抗癌剤SMANCSは，単剤動注としては35.9%と高い奏効率が得られている[3]。しかし，血管閉塞や肝萎縮などの合併症の頻度が高いため，その使用は限られているのが現状である。投与量を制限したり，区域レベル以下の選択的投与を行うなどの対策が必要である。

HCCに対する動注療法の主な成績を表1に示す。単剤および多剤併用いずれも奏効率に大きな幅があり，一概に比較は難しい。現状では単剤動注よりもリザーバー動注による多剤併用療法の成績が若干良好の印象があるが，どの組み合わせが最も効果的か，また本当に多剤併用が単剤に比し優れているかは対象症例の腫瘍進行度や宿主背景の不統一性もあり，後方視的に比較評価することは難しい。ましてや動注療法の延命効果については，対照群との無作為比較試験でしか実証し得ないが，本邦で施行するのは極めて困難な状況である。様々なレジメが試みられるなか，リザーバー動注によるlow-dose FP療法および5-FU/IFN併用療法の成績が注目されている。また最近，新たにone-shot動注用のCDDP製剤が保険認可され，その有用性に期待がもたれる。

III. Low-dose FP 療法

5-FU を effector (key drug), 少量 CDDP を modulator としてリザーバーより併用投与する方法である。5-FU の抗腫瘍効果の機序の一つとして, 細胞内代謝産物 FdUMP によるチミジン合成酵素 (TS) 阻害にて DNA 合成が阻害される。一方, CDDP は腫瘍細胞膜に作用しメチオニンの細胞内への移送が阻害する。その結果, 細胞内メチオニンプールが減少し細胞内でメチオンニン合成酵素が誘導される。それにカップリングする葉酸系代謝が亢進し 5, 10-CH_2-FH_4が増加するため 5-FU の効果が増強する。また, CDDP は蛋白結合型の total platin の状態で血中に長期存在しながら modulator 作用を発揮する。投与方法は, CDDP 10 mg/1 時間および 5-FU 250 mg/5 時間の 5 日間連続投与 2 日間休薬を 4 週間施行し 1 クールとし, これを反復する。low-dose FP 療法の治療成績は総じて良好で, 進行 HCC (n=45) において奏効率 53.3%, 1 年生存率 84%[4], Vp 2 以上の門脈腫瘍栓を伴う HCC (n=48) において奏効率 48%, 1 年生存率 45%[5]などが報告されている。また, 進行 HCC に対して low-dose FP 療法をベースにした多剤併用の有効性も示唆されている。たとえば, low-dose FP 療法に leucovorin を加えた場合の奏効率 (n=9) は 56%で, low-dose FP 療法 (n=10) の 20%に比べて有意に高い[6]。当施設では Vp 2 以上の門脈浸潤を伴う多発進行 HCC (n=9) において, low-dose FP 療法に doxorubicin を加えた FAP 療法を行うことにより, CR 1 例を含む奏効率 44%を得ている[7]。

IV. IFN 併用 5-FU 動注化学療法

IFN が抗癌薬の作用を修飾することが報告され, 各種抗癌薬の全身あるいは動注療法との併用が試みられている。IFN-α 単剤でも抗腫瘍効果として細胞障害, 細胞周期遅延, 癌抗原の発現上昇, NK 細胞・マクロファージ・T 細胞系活性化などの作用が報告されているが, 肝細胞癌に対する単剤での有効性は疑問視されている[8]。当施設では 1997 年より, 既存の治療法で奏効が期待できない高度門脈浸潤 HCC (Vp 3/4) を対象に IFN-α/5-FU 併用療法を行い, 初期成績として奏効率 63% (n=8) を報告している[9]。この IFN-α/5-FU 併用療法の作用機序については, ① IFN 受容体を介した直接的抗腫瘍効果, ② 免疫担当細胞を介した間接的抗腫瘍効果, ③ 血管新生抑制効果, などの関与が考えられている。現在のプロトコールでは, 5-FU は 500 mg/日の 5 日間投与・2 日間休薬を 2 週間施行, 2 週間休薬を 1 クールとして持続動注する。同時に IFN-α を 5×10^6単位/回, 3 回/週, 4 週間を 1 クールとして皮下投与する。また, 副作用や動注による肝障害を考慮して, 適応条件を 70 歳未満, T-Bil 正常, GOT・GPT 100 IU/l 未満, 血小板数 8×10^4/mm^{3}以上, 血清クレアチニン 1.5 mg/dl 以下, PS (0,1) としている。門脈内腫瘍栓に全肝多発病変を伴う最近の症例 (n=40) では奏効率 47.5%, 1 年生存率 50%を得ている[10]。

V. 動注用 CDDP 製剤

CDDP は泌尿生殖器系癌, 頭頸部癌, 非小細胞肺癌, 神経芽細胞腫, 食道癌, 胃癌などに対する点滴静注用抗癌剤として既承認の白金錯体化合物である。CDDP は癌細胞内の DNA 鎖と結合し, DNA 合成とそれに引き続く癌細胞分裂を阻害することにより, 殺細胞効果を発揮し, その抗腫瘍効果は濃度依存性である。肝細胞癌に対する CDDP の感受性も注目され, 国内では静注療法[11]における奏効率 9%, 動注療法[12]における奏効率 19%が報告されており, 局所投与である動注療法のほうが優れた傾向を示している。しかし, 従来の点滴静注用製剤の肝動脈内投与は, 保険適応外使用である上, 低濃度 (0.5 mg/ml)・大容量のため注入時間を要し, 肝動注療法には適さないという問題もあった。そこで, 濃度依存性による殺細胞効果を高め, かつ注入時間の短縮を図るべく, より高濃度 (約 1.5 mg/ml)・少容量に調整可能な微粉末化 CDDP 製剤 (動注用アイエーコール・日本化薬㈱) が肝動注用として開発され, 2004 年 7 月に保険適応に認可された。本剤開発時の後期第 II 相臨床治験では, 至適用量 65 mg/m^{2}での one-shot 反復動注の奏効率は 33.8%であった。単剤動注の成績としては TACE で最頻用される epirubicin の奏効率 15.1%を大きく上回っており[13], 動注用 CDDP は高い有効性が期待できるが, 30%台の奏効率自体は臨床的に十分とはいえず, さらなる投与法の工夫が必要と思われる。これまでにも静注用製剤を施設独自に粉末化して Lipiodol 混合液を作製したり[14], ゼラチン・スポンジを用いて塞栓術と併用するなど報告されている[15]が, 投与方法や至適投与量について一定の見解はない。また, リザーバー・システムからの少量連続投与については, 現在 1 本 100 mg のバイアル製剤しかないため必ずしも適さない。

まとめ

現在, 肝内多発や門脈浸潤を伴う進行 HCC が肝動注療法の主な対象と考えられるが, その適応は肝予備能や全身状態に左右される。最近は 5-FU を key drug とした low-dose FP 療法や IFN 併用療法が高く注目されているが, いずれもリザーバーの使用が前提で, 良好な肝内薬剤分布や血管開存性が重要な要件である。また, 新

たな動注用CDDP製剤の登場も，動注の適応を広げる可能性がある。一方，動注の長期成績は併存する肝硬変の関与も大きいため，延命に寄与したデータは得られておらず，いずれのレジメも標準的治療と評価される段階には至っていない。今後も多様化が予想される動注療法の真価を得るには，科学的な臨床試験による検証が必要と考えられる。

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Elevated Expression of Valosin-Containing Protein (p97) in Hepatocellular Carcinoma Is Correlated With Increased Incidence of Tumor Recurrence

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Purpose: Valosin-containing protein (VCP; also known as p97) has been shown to be associated with antiapoptotic function and metastasis via activation of the nuclear factor-κB signaling pathway. In this study, association of VCP expression with recurrence of hepatocellular carcinoma (HCC) and patient survival was examined.

Patients and Methods: VCP expression in 170 patients (139 male and 31 female) with ages ranging from 31 to 81 years (median, 61 years) was analyzed by quantitative reverse-transcription polymerase chain reaction (RT-PCR) and immunohistochemistry, in which staining intensity in tumor cells was categorized as weaker (level 1) or equal to or stronger (level 2) than that in endothelial cells.

Results: Immunohistochemically, 57 patients (35.2%) showed level 1, and 105 patients (64.8%) showed level 2, VCP expression. Quantitative RT-PCR analysis revealed higher VCP mRNA expression in level 2 patients (n = 7) than level 1 (n = 4) ($P < .05$). Patients with VCP-level 2 HCC showed higher rate of portal vein invasion in the tumor ($P < .01$) and poorer disease-free and overall survival ($P < .0001$ and $P < .05$, respectively) compared with level 1 patients. Multivariate analysis revealed VCP expression level, tumor multiplicity, and degree of fibrosis in the noncancerous liver tissue to be independent prognosticators for disease-free and overall survival. VCP level was an indicator for disease-free survival in each early- (I and II) and advanced- (III and IV) stage group of pathologic tumor-node-metastasis classification ($P < .001$ and $P < .01$, respectively).

Conclusion: VCP expression level has prognostic significance for disease-free and overall survival of patients with HCC.

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HEPATOCELLULAR CARCINOMA (HCC) is one of the most common cancers worldwide, especially in Southeast Asia and Africa.[1] The annual incidence rate of HCC in Japan is approximately 30 per 100,000 population, and its mortality is ranked third as a cancer death.[1] Cirrhosis and chronic hepatitis caused by hepatitis B or C viral infection have been discussed in association with the development of HCC.[2-6] Surgical resection is the main modality of treatment for HCC, but the prognosis remains poor even in curatively resected cases: 5-year survival rate is 25% to 50% after surgery, mainly owing to the high recurrence rate.[7-11]

Several histologic factors have been reported to be prognosticators for HCC. Among them, tumor size and vascular invasion, either portal or hepatic, were the main factors for tumor recurrence[7-15]; these two factors, together with the multiplicity of the tumor, were included in the tumor-node-metastasis (TNM) classification for HCC.[16] However, prognoses of patients with solitary and small-sized tumors without microscopic vascular invasion were still unfavorable.[17] Therefore, it is important to identify other prognostic factors responsible for the recurrence of HCC.

Recently, we identified the gene-encoding valosin-containing protein (VCP; also known as p97) as being associated with metastasis of the murine osteosarcoma cell line by using the mRNA subtraction technique.[18] VCP, a member of the ATPases associated with various cellular activities (AAA) superfamily, is involved in the ubiquitin-dependent proteasome degradation pathway of inhibitor κBα (IκBα), an inhibitor of nuclear factor-κB (NFκB).[19] Cell lines transfected with VCP show the constant activation of NFκB, rapid degradation of phosphorylated IκBα (*p*-IκBα), decreased apoptosis rates after tumor necrosis factor alpha stimulation, and increased metastatic potential.[18] NFκB signaling is suggested to play an important role in proliferation and prevention of apoptosis of hepatocytes during liver regeneration.[20]

In this study, expression level of VCP in HCC was examined by reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemical analysis, and its correlation with recurrence and survival of patients with HCC was evaluated.

PATIENTS AND METHODS

Patients

One hundred seventy patients who underwent curative resection for primary HCC at the Gastroenterological Surgery Division of Osaka University Hospital in Osaka, Japan, during the period from July 1987 to February 2000 were selected for this study. There were 139 male and 31 female patients, with ages ranging from 31 to 81 years (median, 61 years). Thirty-seven patients were positive for hepatitis B virus surface antigen, whereas 99 patients were positive for hepatitis C virus antibody. Preoperative diagnostic imagining examinations, including ultrasonography, computed tomography scan, and angiography, were performed in all patients. Liver function was assessed by combined findings of Pugh-Child's classification, liver biochemistry including serum alpha-fetoprotein (AFP) level, and indocyanine green retention test. Preoperative transarterial embolization was performed in 62 patients (36%). According to the number of resected anatomic segments,[21] surgical procedures used were classified as

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follows: limited resection (79 patients), subsegmentectomy (41 patients), segmentectomy (29 patients), lobectomy (16 patients), and extended lobectomy (five patients).

Surgically resected specimens were fixed in 10% formalin and routinely processed for paraffin embedding. Histologic sections cut at 4-μm thickness were stained with hematoxylin and eosin and reviewed by one of the authors (Y.H.) to determine the following categories: differentiation of tumor cells based on the criteria proposed by Edmondson and Steiner (I, well-differentiated; II, moderately differentiated; III, poorly differentiated; IV, undifferentiated),[2] pattern of growth (expansive or infiltrative), formation of fibrous capsule around the tumor, portal vein invasion, tumor multiplicity, and positivity for the surgical margin. When tumor cells were present within 5 mm from the edge, surgical margin was defined as positive. Degree of inflammation and fibrosis in noncancerous hepatic tissues were shown as the histologic activity index (HAI) score.[22] Regarding inflammatory grade, there were 13 patients with no activity (score of 0), 60 with minimal activity (score of 1 to 3), 84 with moderate activity (score of 4 to 7), and 13 with severe activity (score of $\geq$ 8). The degree of fibrosis was categorized as stage 0 (no fibrosis) in 14 patients, stage 1 (mild fibrosis) in 53, stage 3 (severe fibrosis) in 55, and stage 4 (cirrhosis) in 48.

After surgery, measurement of serum AFP level and ultrasonography and computed tomography scan were performed at 1- and 3-month intervals, respectively. When tumor recurrence was suspected, angiography was performed. The patients were observed until March 31, 2002; the follow-up periods for survivors ranged from 5 to 136 months (median, 43 months) after surgery.

Immunohistochemical Analysis

Immunoperoxidase procedure (avidin-biotin-complex method) was performed on paraffin-embedded sections. Antigen retrieval was performed with heating the sections in 10 mmol/L citrate buffer for 5 minutes. Anti-VCP (p97) monoclonal antibody (PROGEN Biotechnik, Heidelberg, Germany) was used as the primary antibody at a dilution of 1:3,000. Sections were counterstained lightly with methyl green. For negative controls, nonimmunized mouse immunoglobulin G (Vector Laboratories, Burlingame, CA) was used as a primary antibody. Positive staining in endothelial cells in the noncancerous areas in each specimen was used as internal positive control. Stained sections were evaluated in a blinded manner without prior knowledge of the clinicopathologic parameters. Staining intensity in the cytoplasm of the tumor cells was shown in comparison to that of endothelial cells and categorized as follows: weaker (level 1) or equal to or stronger (level 2) than that of endothelial cells. When staining intensity of the tumor cells was different among areas in the same specimen, the predominant pattern was chosen. Cases with negative endothelial cell staining were regarded as having poor antigen preservation and were excluded from further analysis.

Quantitative RT-PCR Analysis of VCP

Total RNA was extracted from fresh frozen samples in 11 cases of HCC with TRIzoL reagent (Invitrogen, Carlsbad, CA). Ten micrograms of DNase I-treated total RNA was used for reverse transcription with Superscript II (Invitrogen). An aliquot representing 100 ng of input RNA was amplified by quantitative real-time PCR using TaqMan PCR Reagent Kit (Applied Biosystems, Foster City, CA) with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) as follows: 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles at 95°C for 15 seconds and 60°C for 1 minute.[23,24] Forward primer 5′-TCACCCACACTGTGCCCATCTACGA-3′, reverse primer 5′-CAGCGGAACCGCTCATTCGCCAATGG-3′, and probe 5′-6-carboxyfluorescein (FAM)-ATGCCC-6-caboxytetramethylrhodamine (TAMRA)–CCCCCATGCCATCCTGCGTp-3′ was used for amplification of beta-actin, and forward primer 5′-AAACCGTGGTAGAGGTGCCA-3′, reverse primer 5′-CTTGGAAGGTGTCATGCCAA-3′, and probe 5′-(FAM) CAGTATCCTGTGGAGCACCCAGACAAATTC (TAMRA)-3′ for VCP. RNA extracted from noncancerous hepatic sample in one case was used as a standard. After reverse transcription, standard cDNA was serially diluted to obtain five standard solutions for use in PCR reaction to generate the reference curve. Relative amount of cDNA in each sample was measured by interpolation in the standard curve,[24] and then relative ratio of VCP/beta-actin expression was calculated for each HCC sample.

Statistics

Statistical analysis was performed by using JMP software (SAS Institute Inc, Cary, NC). Correlation between expression level of VCP at quantitative RT-PCR and immunohistochemistry was evaluated by one-way analysis of variance. Correlation between VCP expression at immunohistochemistry and clinicopathologic parameters was evaluated by using the χ^2 test and Fisher's exact probability test. Overall and disease-free survival rates were calculated by using the Kaplan-Meier method,[25] and difference in survival curves was analyzed by using the log-rank test. Independent prognostic factors were analyzed by the Cox proportional hazards regression model with stepwise

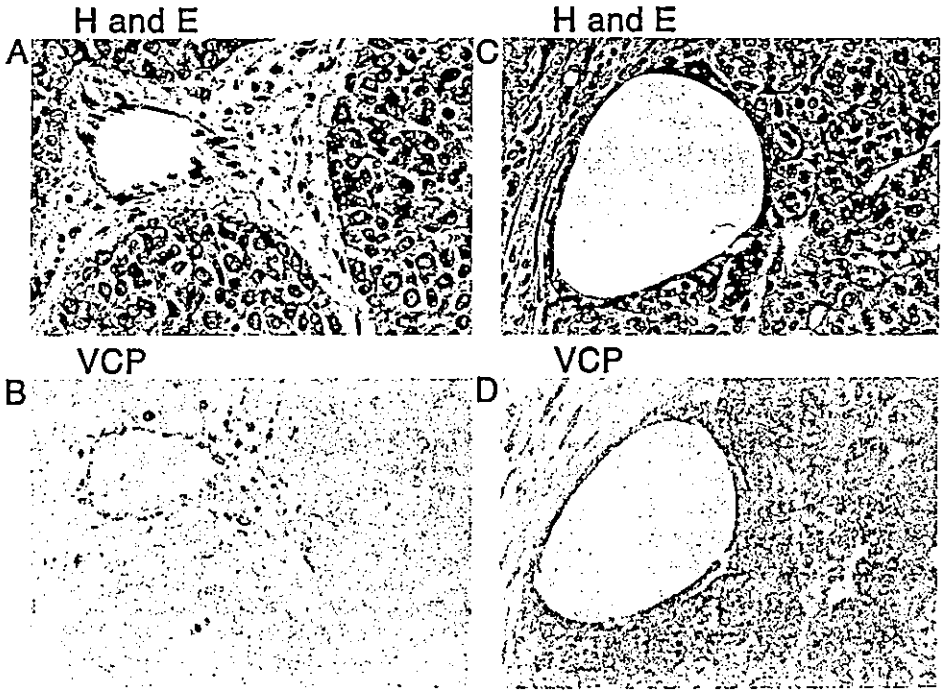

Fig 1. (A, B) Valosin-containing protein (VCP) level 1 hepatocellular carcinoma (HCC). Tumor cells are weakly stained with VCP compared with the endothelial cells. (C, D) VCP level 2 HCC. Tumor cells show strong VCP staining similar to that of the endothelial cells (magnification ×100). H and E, hematoxylin and eosin; VCP, VCP immunohistochemistry.

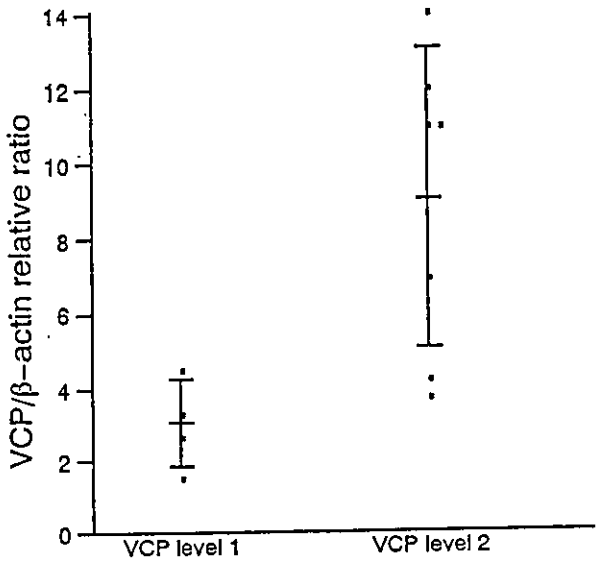

Fig 2. Valosin-containing protein (VCP)/beta-actin relative ratio of mRNA expression in hepatocellular carcinoma with VCP level 1 and 2. All but one case with VCP level 1 showed ratios lower than those of level 2 ($P < .05$). Bars, mean ± SD.

manner.[26] The presence of a statistically significant difference was denoted by $P < .05$.

RESULTS

VCP Expression in HCC

Eight (4.7%) of 170 sections that did not show endothelial staining by immunohistochemistry were regarded as having poor antigen preservation and were excluded from further analysis. The remaining 162 cases showing endothelial staining were evaluated for VCP expression. Forty-two cases showed a constant level 1 staining in the cytoplasm of HCC in every area of the tumor, whereas 15 cases showed level 2 staining at the peripheral zone but level 1 in the larger central area of the tumor. In total, 57 cases (35.2%) were regarded as having level 1 VCP expression. The remaining 105 cases (64.8%) showed level 2 staining throughout the tumors (Fig 1).

Quantitative RT-PCR analysis was performed in four HCCs with level 1 and seven with level 2 expression. Relative ratio of VCP/beta-actin expression in cases with level 1 and 2 expression was 3.1 ± 1.3 and 9.0 ± 4.0 (mean ± SD), respectively ($P < .05$; Fig 2). All but one of level 1 cases showed the lower VCP expression than level 2 cases at the RT-PCR analysis.

Uni- and Multivariate Analyses for Prognostic Factors in HCC

Significant difference was observed in the presence of portal vein invasion between HCC with VCP level 1 (40.0%) and level 2 (61.9%) expression ($P < .01$; Table 1). There was no significant relation between VCP level and other clinicopathologic factors.

Five-year disease-free and overall survival rates of the 162 patients with HCC were 26.7% and 64.9%, respectively. Tumor recurrence during the course was found in remnant liver in 109 patients, lymph node in four, bone in two, and other organs in three. Prognostic significance of VCP expression was analyzed for disease-free and overall survival. Patients with level 1 HCC

Table 1. Relationship Between Valosin-Containing Protein (VCP) Expression and Clinicopathologic Factors

Factor	Total Cases	Cases With VCP Level 2	P
Age			
≤ 60 years	75	47	NS
> 60 years	87	58	
Sex			
Male	132	86	NS
Female	30	19	
HBsAg			
Positive	32	19	NS
Negative	128	84	
HCVAb			
Positive	96	65	NS
Negative	61	38	
Pugh-Child's classification			
A	133	87	NS
B	24	15	
C	0	0	
AFP			
≤ 200 ng/mL	113	68	NS
> 200 ng/mL	49	37	
TAE			
Performed	101	67	NS
Not performed	61	38	
Types of surgery			
Limited resection	76	48	NS
Subsegmentectomy	40	26	
Segmentectomy	27	15	
Lobectomy	15	12	
Extended lobectomy	4	4	
Surgical margin			
Negative	150	98	NS
Positive	12	7	
Tumor size			
≤ 2 cm	33	21	NS
> 2 cm	129	84	
Tumor multiplicity			
Solitary	98	58	NS
Multiple	64	47	
Differentiation			
I	21	12	NS
II	68	40	
III	51	36	
IV	22	17	
Pattern of tumor growth			
Expansive	122	77	NS
Infiltrative	40	28	
Formation of fibrous capsule			
Present	134	85	NS
Absent	28	20	
Portal vein invasion			
Present	88	65	< .01
Absent	74	40	
Stage (pTNM)			
I	25	14	NS
II	80	50	
III	34	22	
IV	23	19	
Inflammatory status (HAI index)			
Absent (0)	13	9	NS
Mild (1-3)	54	31	
Moderate (4-7)	82	53	
Severe (≥ 8)	13	12	
Degree of fibrosis (HAI index)			
Absent (0)	14	8	NS
Mild (1)	52	35	
Severe (3)	49	35	
Cirrhosis (4)	47	26	

Abbreviations: HBsAg, hepatitis B virus surface antigen; HCVAb, hepatitis C virus antibody; AFP, alpha-fetoprotein; TAE, transarterial embolization; pTNM, pathologic tumor-node-metastasis system; HAI, histologic activity index; NS, not significant.

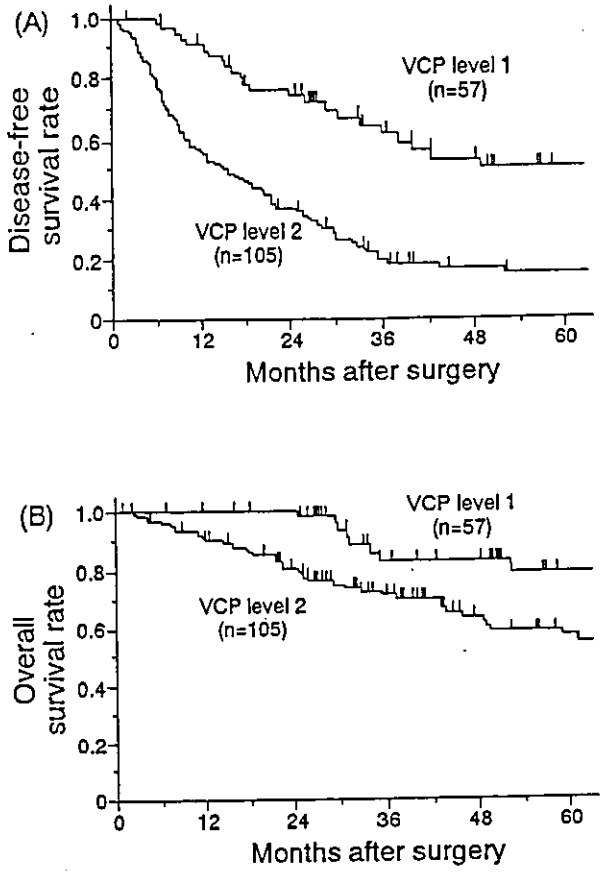

Fig 3. Disease-free (A) and overall (B) survival of patients with valosin-containing protein (VCP) level 1 and 2 hepatocellular carcinoma. Significant difference was observed between the two groups (A, $P < .0001$; B, $P < .05$).

had better 5-year survival rates than those with level 2 HCC (disease-free survival rate, 50.5% *v* 15.3%; $P < .0001$; overall survival rate, 79.2% *v* 57.0%; $P < .05$; Fig 3; Table 2). Twenty-eight (49.1%) of 57 level 1 patients developed recurrent HCC at 32.0 ± 4.2 (mean ± SD) months after the surgery, whereas 90 (85.7%) of 105 level 2 patients developed recurrent HCC at 19.1 ± 2.3 months ($P < .01$).

Serum AFP level, tumor multiplicity, presence of portal vein invasion, degree of fibrosis in the noncancerous liver tissue (HAI index of 0 *v* 1 to 4 and 0 to 1 *v* 3 to 4), and pathologic TNM (pTNM) stage were significant factors for disease-free and overall survival (Table 2). Inflammatory status in the noncancerous liver tissues (two categorizations, HAI index of 0 to 3 *v* > 3 and 0 to 7 *v* > 7) significantly affected disease-free survival but did not affect overall survival.

Multivariate analysis was performed with factors proven to be significant in the univariate analysis, revealing VCP expression level, tumor multiplicity, and degree of fibrosis in the noncancerous liver tissue (different categorizations, HAI index of 0 *v* 1 to 4 for disease-free; 0 to 1 *v* 3 to 4 for overall) to be independent prognostic factors for disease-free and overall survival.

Prognostic Significance of VCP Expression in pTNM Classification

Prognostic significance of VCP expression was further analyzed in patients with HCC according to the pTNM classification system.[19] There was a significant difference in disease-free survival between patients with VCP level 1 and 2 expression in both early (I and II) and advanced (III and IV) stage groups ($P <$ .001 and $P < .01$, respectively; Fig 4).

Table 2. Univariate Analysis of Clinicopathologic Factors for Disease-Free and Overall Survival of Patients With Hepatocellular Carcinoma

Factor and Category	No. of Patients	5-Year Disease-Free Survival (%)	P	5-Year Overall Survival (%)	P
Valosin-containing protein expression					
1: Level 1	57	50.5	< .0001	79.2	< .05
2: Level 2	105	15.3		57.0	
Alpha-fetoprotein					
1: ≤ 200 ng/mL	113	31.1	< .05	70.2	< .01
2: > 200 ng/mL	49	16.8		51.3	
Tumor multiplicity					
1: Solitary	98	38.6	< .0001	72.5	< .001
2: Multiple	64	7.5		52.9	
Portal vein invasion					
1: Present	88	18.9	< .05	56.1	< .05
2: Absent	74	34.5		73.8	
Stage, pTNM					
1: I	25	32.4	< .001	75.8	< .001
2: II	80	37.9	(1-2 *v* 3-4)	68.5	(1-2 *v* 3-4)
3: III	34	4.8	< .05	51.2	< .05
4: IV	23	15.3	(1-3 *v* 4)	60.0	(1-3 *v* 4)
Inflammatory status, HAI index					
1: Absent (0)	13	47.6	< .05	100.0	NS
2: Mild (1-3)	54	27.9	(1-2 *v* 3-4)	66.8	
3: Moderate (4-7)	82	27.8	< .05	62.0	
4: Severe (≥ 8)	13	7.7	(1-3 *v* 4)	55.5	
Degree of fibrosis, HAI index					
1: Absent (0)	14	54.4	< .05	85.7	< .01
2: Mild (1)	52	33.8	(1 *v* 2-4)	75.7	(1-2 *v* 3-4)
3: Severe (3)	49	22.2		64.8	
4: Cirrhosis (4)	47	20.5		51.7	

Abbreviation: HAI, histologic activity index.

Table 3. Multivariate Analysis of Clinicopathologic Factors for Disease-Free and Overall Survival of Patients With Hepatocellular Carcinoma

Factor and Category	Relative Risk	95% Confidence Interval	χ^2	P
Disease-free survival				
VCP expression				
1: Level 1	2.78	1.83-4.36	25.0	< .0001
0: Level 2				
Tumor multiplicity				
1: Solitary	2.51	1.71-3.66	21.9	< .0001
0: Multiple				
Degree of fibrosis in noncancerous liver tissues				
1: Absent of fibrosis (HAI index: 0)	2.65	1.19-7.517	5.96	.0147
0: Mild fibrosis to cirrhosis (HAI index: 1-4)				
Overall survival				
VCP expression				
1: Level 1	2.08	1.19-3.87	6.68	.0097
0: Level 2				
Tumor multiplicity				
1: Solitary	2.32	1.38-3.93	9.98	.0016
0: Multiple				
Degree of fibrosis in noncancerous liver tissues				
1: Absent or mild fibrosis (HAI index: 0-1)	2.20	1.22-4.28	7.01	.0081
0: Severe fibrosis or cirrhosis (HAI index: 3-4)				

Abbreviations: VCP, valosin-containing protein; HAI, histologic activity index.

DISCUSSION

To establish appropriate therapeutic modalities for HCC, precise estimation for tumor recurrence is essential. Although conventional TNM staging, which mainly consists of tumor size, vascular invasion, and tumor multiplicity, is a significant prognosticator for disease-free and overall survival of patients with HCC,[8,10,11] increased recurrence rates among patients even at low stage (I and II) has been reported.[8,10,11] To reinforce the accuracy of prognostication of the TNM staging system, several biologic factors, such as cellular proliferation indices, DNA ploidy pattern in tumor, and aberrant gene expression,[27] have been proposed as candidates for prognosticators. However, the prognostic significance of these factors is still controversial among studies.[27] This study was conducted to clarify whether VCP expression level could be a new prognostic factor for HCC.

Patient characteristics (sex, age, viral association, and 5-year survival rates) in this series were similar to those of previous reports from Japan,[8,11] other Asian,[9,10,12] and Western countries.[7] Uni- and multivariate analyses in this study showed the prognostic significance of tumor multiplicity, serum AFP index, presence of portal vein invasion, pTNM stage, and degree of fibrosis in the noncancerous lesion, as reported previously.[8-12,28] These findings indicate that the results obtained from these cases are applicable to HCC in other countries.

In this study, VCP expression level was examined in 11 cases by combined quantitative RT-PCR and immunohistochemical analyses and in 151 cases by immunohistochemistry alone. In the 11 cases, there was a clear correlation in VCP expression between the protein (immunohistochemistry) and mRNA (RT-PCR) level, which indicates the reliability of immunohistochemistry for evaluation of VCP expression.

Among the clinicopathologic factors examined, a significant correlation was observed between increased VCP expression and the presence of portal vein invasion of the tumor cells, which was in agreement with our previous study, in which we found that VCP overexpression is correlated with increased metastatic potential of tumor cells in the experimental metastasis model.[13] For a successful tumor formation in the portal vein, antiapoptotic

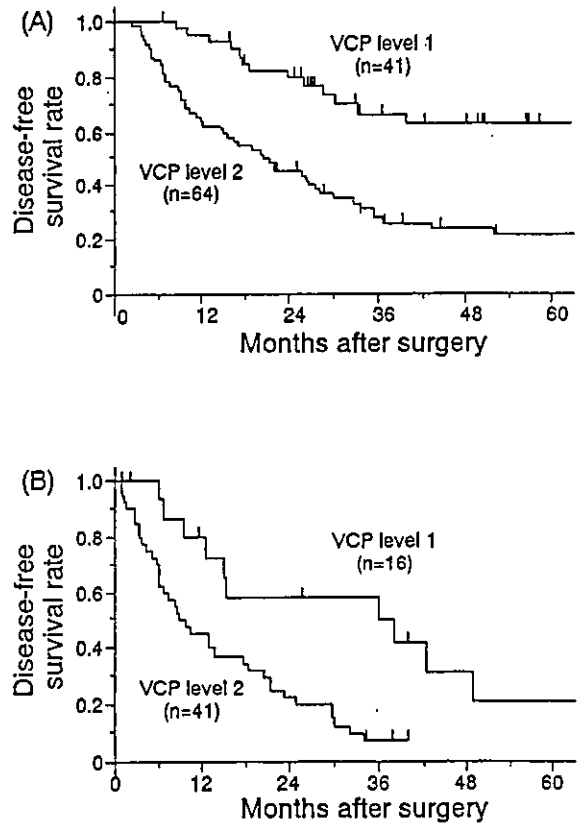

Fig 4. Disease-free survival rates of patients with valosin-containing protein (VCP) level 1 and 2 hepatocellular carcinoma in (A) early (I and II) and (B) advanced (III and IV) pathologic tumor-node-metastasis stages. Significant difference was observed between VCP level 1 and 2 patients in both groups (A, $P < .001$; B, $P < .01$).

pathway including NFκB signaling might be important in preventing cells from developing apoptosis.[18,29] Our previous study indicated that VCP is associated with antiapoptotic function and metastasis via activation of the NFκB signaling pathway.[18] Kirimlioglu et al[30] showed the correlation between decreased apoptotic rate and increased rate of portal vein invasion in HCC. Taken together, the present results show the importance of VCP expression for growth of HCC in the portal vein system.

Uni- and multivariate analyses revealed the VCP expression level to be an independent prognosticator for HCC recurrence and patient survival. In addition, VCP level proved to be a prognosticator for recurrence of the disease in patients at both the early (I and II) and advanced (III and IV) stages of pTNM classification: the 5-year disease-free survival rate in patients with VCP level 1 and 2 was 64.5% and 21.5% at low stage, respectively, and 22.0% and 0% at high stage, respectively. Combination of VCP level and pTNM classification is a useful tool for prediction of prognosis for patients with HCC.

Recently, improvement of prognosis of HCC patients by adjuvant therapies, such as chemotherapy,[31,32] immunotherapy,[33,34] and combined chemo- and immunotherapy[35] has been reported. The grouping system described above could be a valuable guide for choosing various modalities of adjuvant therapy. In patients with early-stage VCP level 1 HCC, favorable outcome could be expected without adjuvant therapy, whereas patients with level 2 and/or advanced-stage HCC should receive intensive treatment with adjuvant therapy.

In conclusion, VCP expression as determined by immunohistochemistry could be used as a new prognosticator for HCC. This study shows that stratification of HCC patients based on the stage of disease, tumor multiplicity, and VCP expression level is useful for prediction of prognosis for HCC patients. This system might open a new way to explore effective modalities of treatment for HCC.

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Overexpression of CDC25A Phosphatase Is Associated with Hypergrowth Activity and Poor Prognosis of Human Hepatocellular Carcinomas[1]

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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[3] 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 β-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_1-S and G_2-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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[3] 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; β-gal, β-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_1 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_2 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 peptides 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 $MgCl_2$, 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

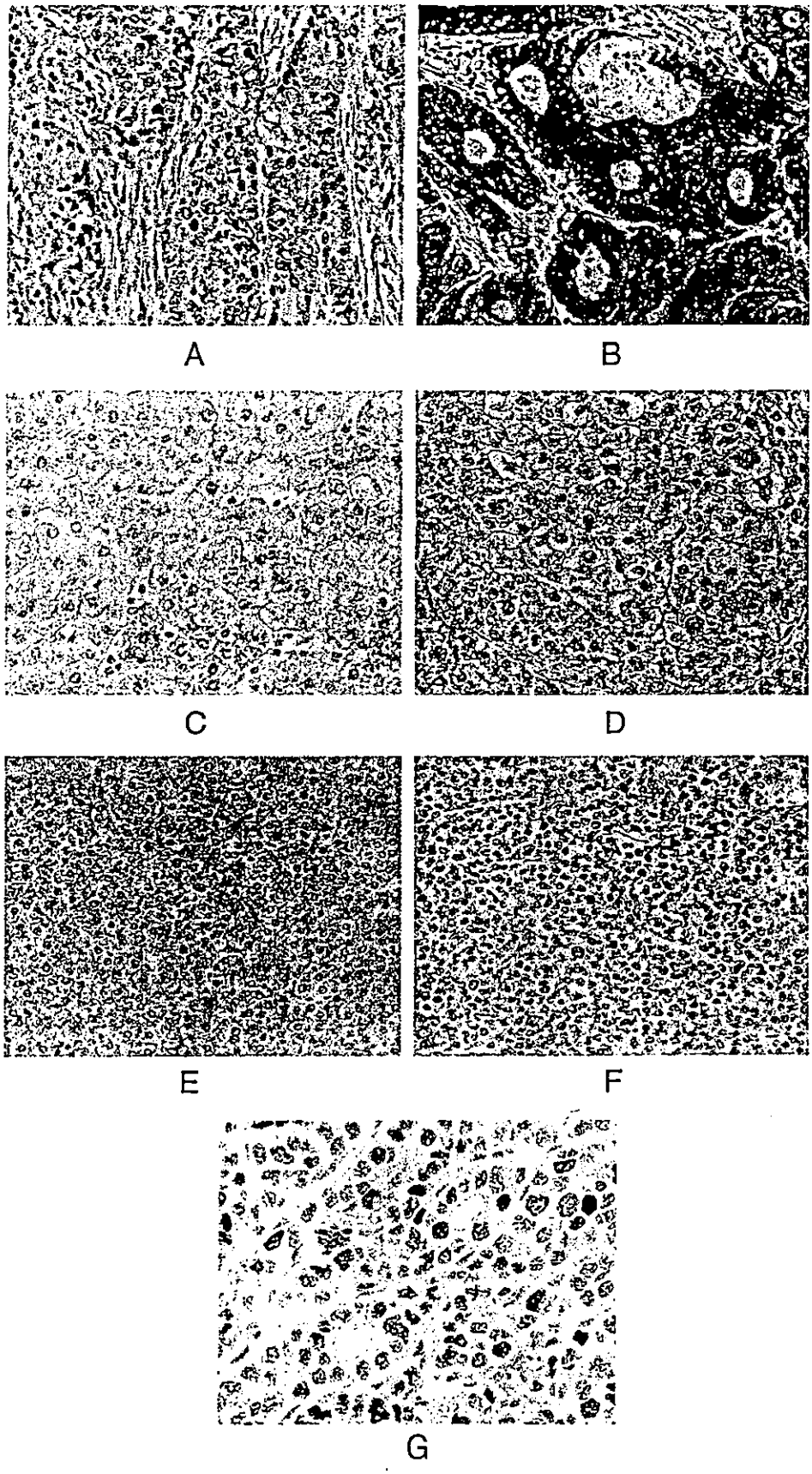

Fig. 1 Immunostaining with antihuman CDC25A and CDC25B antibodies in colorectal carcinoma tissues. *A*, a representative colorectal carcinoma tissue with low expression level of CDC25A. *B*, a representative colorectal carcinoma with high expression level of CDC25B. Immunostaining with antihuman CDC25A 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*, ×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 β-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 *Not*I 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 1×10^5/well in 6-cm dishes. To monitor the transfection efficiency, either the CDC25A plasmid or pcDNA3 vector was cotransfected with a cytomegalovirus β-gal plasmid at a ratio of 2:1 (1 μg:0.5 μg), 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 β-gal staining, β-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.

β-Gal-introduced cells were visualized by incubation with β-gal staining solution (4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM $MgCl_2$, and 0.02% 5-bromo-4-chloro-3-indolyl-β-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 χ^2 test or Fisher's exact probability test. Mean values were compared using the Mann-Whitney test. Data are expressed as mean ± 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).

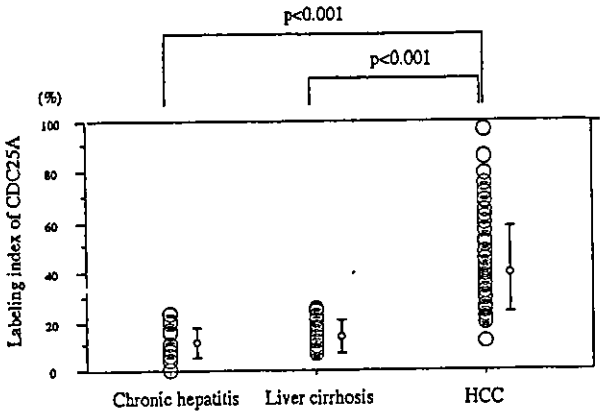

Fig. 2 LI of CDC25A in CH, liver cirrhosis, and HCC. HCC displays significantly higher LI than CH or liver cirrhosis (both for $P < 0.001$); *bars*, ±SD.

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. 1*C*); the LI was 13.5 ± 4.9% in CH-based hepatocytes ($n = 23$) and 15.7 ± 4.3% in cirrhotic livers ($n = 36$). In carcinoma tissues, 25 cases (42.4%) expressed nuclear CDC25A with moderate to strong intensity (Fig. 1*D*), 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 ± 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 β-gal construct was ~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 ~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 (γ^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. 1*E*) and the other half (31 of 59: 52.5%) dis-

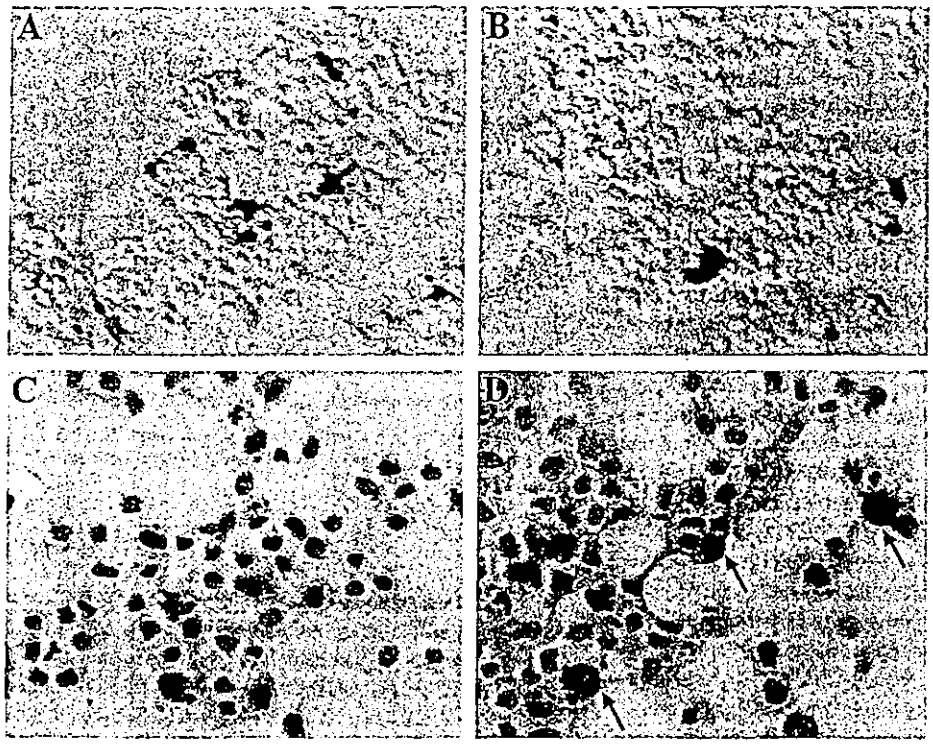

Fig. 3 Transfection assays. Transfection efficiency was ~12% in pcDNA3-transfected cultures (*A*) and 10% in CDC25A-transfected cultures (*B*). β-Gal-positive HEK 293 cells were in *blue*. pcDNA3 transfected cultures did not express nuclear CDC25A (*C*), whereas CDC25A-transfected cultures did display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

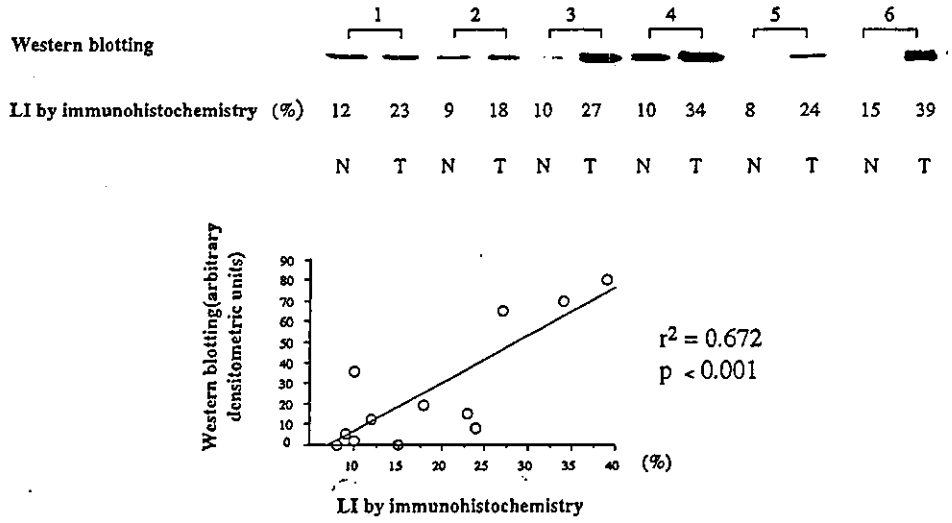

Fig. 4 Western blot analysis for CDC25A. Western blotting was performed on six pairs of HCC and its noncancer counterpart. There was a significant correlation between CDC25A level on immunoblot and LI determined by immunohistochemistry (γ^2) = 0.672; $P < 0.001$).

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. 1*F*) 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. 1*B*. 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 overexpression, CDC25A was overexpressed in 9 of 13 (69%) cases

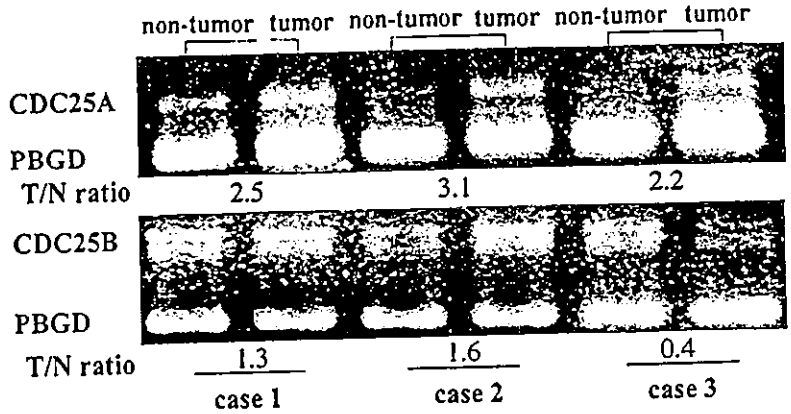

Fig. 5 RT-PCR analysis for CDC25A and CDC25B mRNAs. RT-PCR analysis was performed for CDC25A (*top panel*) or CDC25B (*bottom panel*) mRNA in paired normal and carcinoma tissues. The value of CDC25A or CDC25B band relative to PBGD band was calculated for each sample, and the T:N ratio was determined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

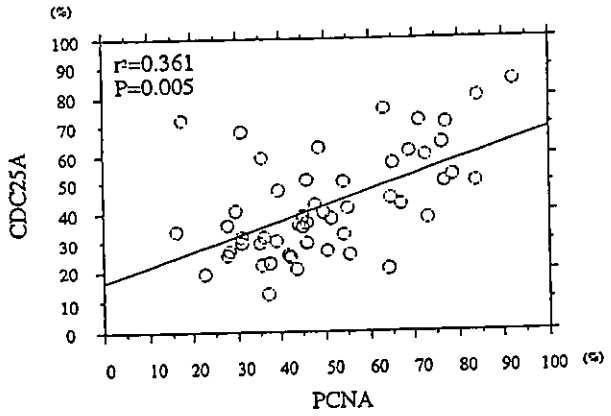

Fig. 6 Relationship between CDC25A and PCNA expression, significant correlation was found ($\gamma^2 = 0.361$; $P < 0.01$).

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 ± 14.8%, which was significantly higher than 20.5 ± 7.2% in CH-based hepatocytes, and 15.5 ± 6.0% in cirrhotic hepatocytes ($P < 0.001$ for both; Fig. 1*G*). 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 Low	CDC25A High	*P*
Age	≧60	49	23	26	N.S.[a]
	<60	10	3	7	
Gender	Male	49	23	26	N.S.
	Female	10	3	7	
Tumor size	<2 cm	18	5	13	N.S.
	≧2 cm	41	21	20	
Histological grade[b]	Well/mod.	38	23	15	0.001[c]
	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[c]
	No	50	25	25	
Number of tumor[d]	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[e]	Yes	21	7	14	N.S.
	No	25	15	10	

[a] N.S., not significant.
[b] Mod., moderately; poor., poorly; undiff., undifferentiated.
[c] Statistically significant.
[d] This category includes both intrahepatic metastasis and tumors generated by multicentric carcinogenesis.
[e] 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

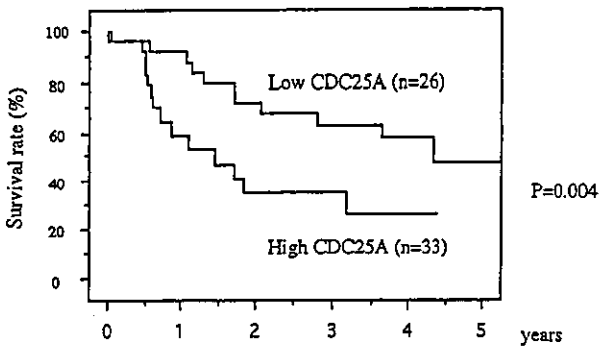

Fig. 7 Kaplan-Meier curves for DFS according to CDC25A expression in 59 patients with HCC. High expression of CDC25A was significantly associated with worse prognosis ($P = 0.004$).

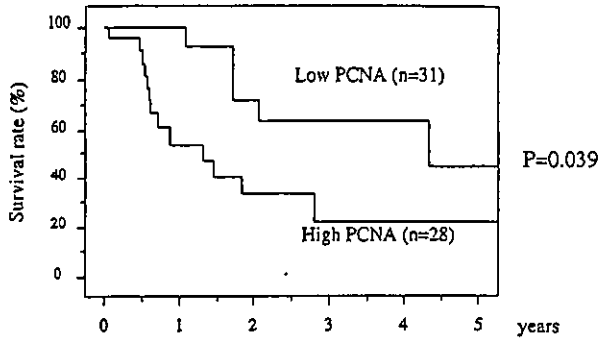

Fig. 8 Kaplan-Meier curves for DFS according to PCNA expression in 59 patients with HCC. High expression of PCNA was significantly associated with worse prognosis ($P = 0.039$).

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 β 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 β 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[a]	*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

[a] 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_1 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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JTE-522, a Cyclooxygenase-2 Inhibitor, Is an Effective Chemopreventive Agent Against Rat Experimental Liver Fibrosis

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Background & Aims: The aim of this study was to assess the effects of cyclooxygenase (COX)-2 inhibition on rat experimental liver fibrogenesis. *Methods:* We investigated the inhibitory effects of a selective COX-2 inhibitor, JTE-522, on liver fibrosis induced by a choline-deficient, L-amino acid–defined diet (CDAA). Inhibitory effect was also tested in a second model of thioacetamide (TAA)-induced liver fibrosis. *Results:* CDAA induced liver fibrosis and preneoplastic foci at 12 weeks and cirrhosis at 36 weeks. Hepatocellular carcinoma was noted in 13 of 15 rats (87%). JTE-522 significantly inhibited fibrosis and development of preneoplastic lesions in a dose-dependent manner and completely inhibited generation of cirrhosis and hepatocellular carcinoma at both low and high doses (10 and 30 mg/kg body wt/day, respectively). JTE-522 administrated only from 12 weeks to 36 weeks also prevented cirrhosis and formation of hepatocellular carcinoma. JTE-522 itself did not cause local or systemic gross or histopathologic changes at 36 weeks. Mechanistic studies indicated that the CDAA model displayed up-regulation of several biomarkers, including COX-2, arachidonate metabolite (prostaglandin E_2), serum aspartate aminotransferase, and c-myc expression. The model also showed an increased proportion of activated hepatic stellate cells, proliferating cell nuclear antigen index, and CD45-positive inflammatory cells in the liver. JTE-522 effectively diminished these changes. JTE-522 exhibited similar antifibrosis effects in the TAA model. *Conclusions:* Our results suggest that COX-2 is involved in CDAA- and TAA-induced liver fibrosis. Our data also indicate that JTE-522 is a potent chemopreventive agent of rat liver fibrosis with low toxicity.

Primary hepatocellular carcinoma (HCC) is one of the most common tumors in Southeast Asia and Africa. The prognosis of HCC is generally poor, and the 5-year survival rate is limited to 25%–49% after surgery. In the United States, it has been estimated that 400,000 people have cirrhotic liver disease or cirrhosis; 13,900 new cases of HCC were diagnosed in 1998.[1] Most patients diagnosed with HCC have underlying liver cirrhosis commonly caused by infection with hepatitis C virus. Similarly, hepatitis C virus–based liver cirrhosis is a serious problem in Japan because it develops to HCC at a rate of 5%–7% per year.[2] Therefore, an effective strategy to prevent cancer development is strongly desired. Because fibrosis is a prominent event that largely increases from hepatitis to cirrhosis, an effective strategy might be to prevent progression of liver fibrosis.

Cyclooxygenase (COX) is a rate-limiting enzyme involved in the conversion of arachidonic acid to prostaglandin (PG) H_2, the precursor of various compounds including PGs, prostacyclin, and thromboxanes. Two COX genes, COX-1 and COX-2, have been identified that share more than 60% identity at the amino acid level.[3] COX-1 is constitutively expressed in many tissues and is responsible for various physiologic functions, including cytoprotection of the stomach, vasodilatation in the kidney, and production of a proaggregatory prostanoid, thromboxane A_2, by platelets. On the other hand, COX-2 is an inducible immediate-early gene and was originally found to be induced by various stimuli, such as mitogens and cytokines, and growth factors.[4] Overexpression of COX-2 has been reported in various chronic inflammatory diseases.[5]

Abbreviations used in this paper: CDAA, choline-deficient, L-amino acid–defined diet; COX, cyclooxygenase; CSAA, choline-supplemented, L-amino acid–defined diet; GST-P, glutathione S-transferase placental form; PCNA, proliferating cell nuclear antigen; PCR, polymerase chain reaction; α-SMA, α-smooth muscle actin; TAA, thioacetamide.

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In recent years, we and other investigators have shown increased COX-2 expression in various types of human cancers and precursor lesions.[6–9] The relevance of COX-2 in human neoplasia has been best proven in patients with familial adenomatous polyposis. Double-knockout mice for the *APC* and *COX-2* genes showed marked reduction in the size and frequency of intestinal polyps, and studies using COX-2 inhibitors inhibited similar results in Apc knockout mice.[10,11] Finally, celecoxib, a selective COX-2 inhibitor, significantly inhibited growth and genesis of colon polyps in patients with familial adenomatous polyposis.[12]

In human hepatocarcinogenesis, the above scenario has not yet been completely delineated. We previously showed that COX-2 expression is up-regulated from normal liver to chronic hepatitis and the highest COX-2 expression was noted in liver cirrhosis, which was even higher than the levels in HCC.[8] Other investigators also showed a relatively high COX-2 expression in early HCC but not advanced HCC.[7] These findings suggest that COX-2 may participate in liver fibrogenesis rather than cancer progression. However, evidence on liver fibrosis or HCC in relation to COX-2 is limited, and the functional significance of COX-2 expression in the process of liver fibrosis has not yet been confirmed.

To explore the effects of COX-2 inhibition against liver fibrosis and cirrhosis as well as HCC, we conducted in vivo animal studies using a novel selective COX-2 inhibitor, JTE-522 (4-(4-cyclohexyl-2-methyloxazol-5-yl)-2-fluorobenzenesulfonamide), which at 100 μmol/L is known to selectively inhibit in vitro COX-2 activity without affecting COX-1.[13,14] Previous studies have shown that JTE-522 inhibits polypogenesis in Apc knockout mice, hematogenous metastasis of colon cancer cells, and N-nitrosomethylbenzylamine (NMBA)-induced esophageal tumorigenesis in various animal models.[15–17] To induce liver cirrhosis in rats, we used a choline-deficient, L-amino acid—defined diet (CDAA).[18,19] A choline-deficient diet is a primeval carcinogen for rat hepatocarcinogenesis, and CDAA is a choline-deficient modified diet (choline-deficient diet plus L-amino acid) that exerts more potent carcinogenicity. This model of liver cirrhosis displays 2 phenomena: (1) formation of liver fibrosis and preneoplastic foci at the early phase, and (2) development of cirrhosis and HCC at the late phase. To confirm the effects of JTE-522 noted in CDAA-fed rats, we also used a second model of rat liver fibrosis induced by thioacetamide (TAA).[20]

Materials and Methods

Animals, Diets, and COX-2 Selective Inhibitor

A total of 116 male 5-week-old Wistar rats weighing 150–153 g were purchased from Shionogi Co. (Osaka, Japan). They were housed in an air-conditioned room maintained at 25°C with a 12-hour dark/light cycle. The control diet (choline-supplemented, L-amino acid—defined diet [CSAA], no. 518754) and CDAA (no. 518753) were obtained from Dyets Inc. (Bethlehem, PA). The amino acid composition of the CDAA and CSAA was made up only of pure L-amino acids with the exception of glycine. For the TAA-induced liver fibrosis model, regular laboratory chow diet was used. The selective COX-2 inhibitor, JTE-522, was obtained from Japan Tobacco, Inc. (Tokyo, Japan).[13,14] JTE-522 was dissolved in ethanol and premixed with CDAA or chow diet once per month by Central Laboratories for Experimental Animals Inc. (Tokyo, Japan) and stocked at 4°C in the dark. Diets were replaced every week, and body weight was monitored weekly.

Table 1. Experimental Protocol

Experiment	n	Length of experiment (*wk*)
A		
CDAA alone	5	12
CDAA + JTE-522 (10 mg · kg^{-1} · day^{-1})	5	12
CDAA + JTE-522 (30 mg · kg^{-1} · day^{-1})	5	12
CSAA alone	5	12
B		
CDAA alone	5	36
CDAA + JTE-522 (10 mg · kg^{-1} · day^{-1})	10	36
CDAA + JTE-522 (30 mg · kg^{-1} · day^{-1})	10	36
CSAA alone	10	36
CSAA + JTE-522 (30 mg · kg^{-1} · day^{-1})	10	36
C		
CDAA + JTE-522 (10 mg · kg^{-1} · day^{-1})	10	CDAA (12)/JTE-522 in CDAA (12–36)
CDAA + JTE-522 (30 mg · kg^{-1} · day^{-1})	10	CDAA (12)/JTE-522 in CDAA (12–36)

Experimental Protocols

The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Osaka University.

CDAA model. Table 1 summarizes the protocols for the CDAA model. Experiment A was designed as a 12-week protocol to examine the effects of JTE-522 on early pathologic stages, at which significant liver fibrosis and preneoplastic foci are known to appear.[18,19] This experiment included 4 groups of 5 rats each. Group 1 received CDAA alone, groups 2 and 3 received CDAA containing JTE-522 at a dose of either 10 mg · kg^{-1} · day^{-1} or 30 mg · kg^{-1} · day^{-1}, respectively, and group 4 received the control diet (CSAA alone). Experiment B, a 36-week protocol, was designed based on the report that HCC develops after 32 weeks[18,19] to examine the effects of JTE-522 on late events such as formation of