

Expression of β -catenin in Hepatocellular Carcinoma in Relation to Tumor Cell Proliferation and Cyclin D1 Expression

Alteration of β -catenin expression has been implicated in the development of hepatocellular carcinoma (HCC). It has been also reported that β -catenin can influence the tumor cell proliferation or cyclin D1 expression, one of the target factors of β -catenin. We performed an immunohistochemical analysis of β -catenin and cyclin D1 in 77 patients with resected HCCs, and examined the relationships between the expressions of β -catenin and cyclin D1, and other pathologic parameters including the mitotic index. Altered expressions of β -catenin including nonnuclear overexpression and nuclear expression were detected in 58.4% of HCCs (45/77) and showed significant correlations with large tumor size, poor histologic grade, and high tumor stage. The mean mitotic index of HCCs with nuclear expression (3.2 ± 3.0) and nonnuclear overexpression (2.7 ± 2.5) was significantly higher than that of tumors with no overexpression (1.7 ± 1.4) ($p=0.018$ and 0.038 , respectively), however, no correlation was noted between the expressions of cyclin D1 and β -catenin. In addition, nonnuclear overexpression out of two altered expression patterns was more frequent (37.7% versus 20.8%) as well as pathologically more significant than nuclear expression. These results indicate that the altered expression of β -catenin in HCC may play an important role in tumor progression by stimulating tumor cell proliferation, and nonnuclear overexpression may have pathologic significance in HCC.

Key Words : β -catenin; Cell Adhesion Molecules; Cyclin D1; Mitotic index; Immunohistochemistry; Liver Neoplasms; Carcinoma, Hepatocellular

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INTRODUCTION

β -Catenin plays a critical role as a component of the cell-cell adhesion complex and as a coactivator of the T-cell transcription factor/lymphoid enhancer binding factor (TCF/LEF) family of transcription factors (1-5). Inappropriate activation of this pathway leads to an increase of the level of β -catenin, which can translocate into the nucleus and up-regulate the target gene expression and cell proliferation through binding to the TCF/LEF family members (3-5). It is now apparent that altered expression of β -catenin is an important event in the genesis of a number of malignancies (6-8). β -Catenin gene mutation and nuclear accumulation found in hepatocellular carcinoma (HCCs) ranged from 13-34% and 11-43%, respectively, however, the clinical implication of the aberrant β -catenin expression in HCC remains still unclear (9-16).

Cyclin D1 is a major regulator of the progression of cells into the proliferative stage of the cell cycle (17, 18). Recently, cyclin D1 has been reported as one of the target genes of the β -catenin/TCF pathway; a TCF-binding site was identified in the promoter region of cyclin D1, and a significant correlation between the expressions of cyclin D1 and β -catenin has been found in human breast and colon cancers (19-21). Cyclin

D1 gene amplification was reported in less than 15% of HCC patients (22, 23); however, the frequencies of overexpressions of cyclin D1 mRNA and cyclin D1 protein were reported in up to 22% (23) and 58% (24), respectively, in HCC patients. These results suggest that another mechanism inducing the overexpression of cyclin D1 mRNA or protein may be present.

In the present study, to evaluate the role of β -catenin in hepatocarcinogenesis as well as the relationships among the expressions of β -catenin and cyclin D1, and tumor cell proliferation, we performed an immunohistochemical analysis of β -catenin and cyclin D1 in 77 patients with HCCs, and studied the relationships between the expressions of β -catenin and cyclin D1, mitotic index, and other pathologic parameters.

MATERIALS AND METHODS

Patients and Tissue Samples

We retrieved 77 formalin-fixed, paraffin-embedded, surgically resected cases of HCC at Inje University Seoul Paik Hospital, which were collected from 1997 to 2001. Seventy-three (94.8%) of the 77 patients were seropositive for HBsAg

and only 4 (5.2%) had serum anti-HCV. Seventy-two patients were men and 5 were women, and the age of the patients ranged from 35 to 72 yr (mean age, 53 yr). Sixty-seven of the patients (87%) had a precirrhotic or cirrhotic nontumorous liver, and the others showed chronic viral hepatitis.

Pathologic Examination

Conventional pathologic parameters were examined. The histologic grade of tumor differentiation was assigned according to the Edmondson and Steiner grading system (25). After grading, the tumors were classified into 2 groups—well (grade I+II) ($n=37$) and poor (grade III+IV) ($n=40$). The size of the 77 HCCs ranged from 1.0 to 17 cm (mean, 3.7 cm), and the tumor size was classified based on the criteria of Yumoto et al. (26)—small (tumor mass <3 cm) ($n=32$) and large (tumor mass ≥ 3 cm) ($n=45$). The tumor staging was performed by using International Union Against Cancer (UICC, 1997) criteria (27)—stage 1 ($n=7$), stage 2 ($n=27$), stage 3 ($n=24$), and stage 4 ($n=19$), respectively.

Immunohistochemical Staining

The conventional avidin-biotin complex (ABC) method was performed on 4 μ m-thick sections from each lesion. For antigen retrieval, the sections were immersed in citrate buffer and processed in a microwave oven at 95°C for 10 min. Primary monoclonal antibodies of β -catenin (Clone: 14, Transduction Laboratories, Lexington, KY) and cyclin D1 (Clone: A-12, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) were used at a 1 to 100 dilution. Sections were immunostained using the ABC method. 3-Amino-9-ethylcarbazole (AEC) was used as the chromogen and Meyer's hematoxylin as a nucleus counterstain. Normal saline was used as a substitute for the primary negative controls. The β -catenin expression patterns were divided into three groups as follows: 1) Nuclear expression, nuclear staining in more than 20% of the tumor cells with or without cytoplasmic expression; 2) Nonnuclear overexpression, increased membranous and/or cytoplasmic staining with no identifiable nuclear staining in at least 50% of the tumorous areas; 3) No overexpression, weakly membranous staining similar to that of the adjacent nonneoplastic hepatocytes. We also categorized the cases showing nuclear expression or nonnuclear overexpression as altered expression and the cases showing no overexpression as normal. For cyclin D1, only distinct nuclear staining was regarded as positive. The cyclin D1 labeling index (LI) was defined as the average of the percentages of cyclin D1-positive cells on 10 randomly selected high power ($\times 400$) fields. The mitotic index was calculated from the numbers of mitotic cells in 10 random high power fields ($\times 400$). The mean value of the mitotic cells counted in each field was defined as the mitotic index. We applied Ki-67 immunostaining (Clone: 7B11, Zymed Laboratories, San Francisco, CA, U.S.A.) in selected

cases with the same methods.

Statistical Analysis

The β -catenin expression in relation to the pathologic parameters was examined with the χ^2 test. To analyze the statistical differences of cyclin D1 LI and mitotic index between groups, the unpaired Student's *t*-test or ANOVA was used. The correlation between cyclin D1 LI and the mitotic index was analyzed by Pearson's correlation. Significance was defined as $p < 0.05$. All statistical analyses were performed using SPSS software (version 10.0, SPSS INC., Chicago, IL, U.S.A.).

RESULTS

β -Catenin Expression

In the nontumorous areas, hepatocytes showed weak to moderate membranous staining with little cytoplasmic expression, and the bile ductules showed more prominent membranous expression (Fig. 1A). In the HCCs, altered expression, including two distinct patterns of nuclear expression and nonnuclear overexpression, was observed in 45 cases (58.4%). In 16 cases (20.8%) showing nuclear expression, nuclear immunostaining was present in 20–60% of neoplastic cells and considerable intracytoplasmic expression was accompanied (Fig. 1B). In 29 cases (27.7%) showing nonnuclear overexpression, β -catenin expression was localized in the cytoplasm, especially close to the cell membrane, without nuclear expression and the intensity was obviously stronger than those of nonneoplastic hepatocytes (Fig. 1C, D). No overexpression was observed in 32 of the 77 HCCs (41.6%), and none of the tumors was completely negative for β -catenin throughout the tumor. According to associated viral status, altered expression was noted in 44 of 73 HBV-associated tumors (60.3%) and one of 4 HCV-associated tumors (25%).

Tumors with altered expression showed significant correlations with large tumor size ($p=0.027$), poor histologic grade ($p=0.032$), and high tumor stage ($p=0.028$). Tumors with nonnuclear overexpression showed a positive correlation with portal vein invasion ($p=0.006$) and tumor size ($p=0.044$), while nuclear expression showed no correlation with any of pathologic parameters examined (Table 1).

Mitotic Index and Cyclin D1 Labeling Index (Table 2)

The average MI in HCCs was 2.4 ± 2.3 (range 0.1–11). The MI showed a strong correlation with poor histologic grade ($p=0.004$), large tumor size ($p=0.002$), presence of portal vein invasion ($p<0.001$), and high tumor stage ($p<0.001$).

The average cyclin D1 LIs in the nontumorous areas and HCCs were $13.8 (\pm 13.2; \text{range } 1\text{--}48)$ and $26.2 (\pm 18.2; \text{range } 1\text{--}80)$, respectively (Fig. 2A, B). There was a positive corre-

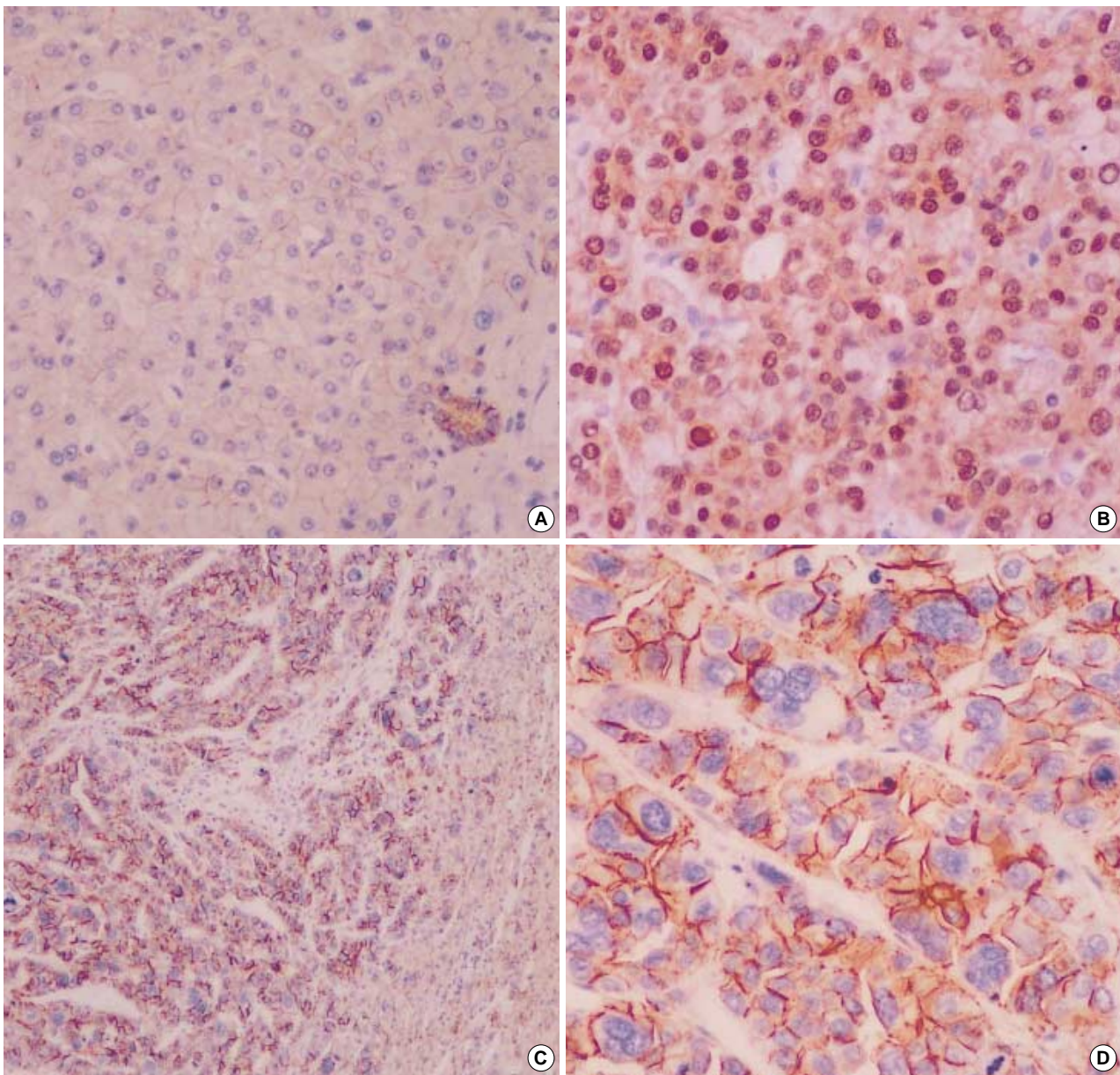


Fig. 1. β -catenin immunohistochemical staining. (A) Nonneoplastic hepatocytes showing homogeneous weak membranous staining. The bile ducts show moderate membranous staining, compared with hepatocytes, (B) HCCs showing strong nuclear and considerable cytoplasmic stainings, and (C, D) Low and high magnification photographs of HCCs showing nonnuclear overexpression when compared to the adjacent nontumorous area. Strong membranous staining with occasional cytoplasmic staining is uniformly distributed throughout the tumor.

lation between cyclin D1 LIs and tumor size ($p=0.02$). However, the cyclin D1 LIs were strikingly variable according to the histologic differentiation, and the average cyclin D1 LIs in HCCs with well-differentiated histologic grade (28.3 ± 17.6) were higher than those in cases with poor histologic grade (24.3 ± 18.7), although statistically not significant.

Interestingly, we observed fifteen cases of HCC with high cyclin D1 expression localized in the central portions of the thick trabeculae or pseudoglandular pattern (Fig. 2C). When we applied Ki-67 immunostaining in these cases, we found

a noticeable difference between the topologic distribution of cyclin D1 and Ki-67 stainings (Fig. 2C, D). Furthermore, the cyclin D1 LIs showed no correlation with the MI ($p=0.298$, Pearson's correlation test).

Relationships of Mitotic Index and Cyclin D1 Labeling Index with β -Catenin Expression

According to β -catenin expression patterns (Table 3), the average MIs (\pm SD) in cases with nuclear expression, nonnu-

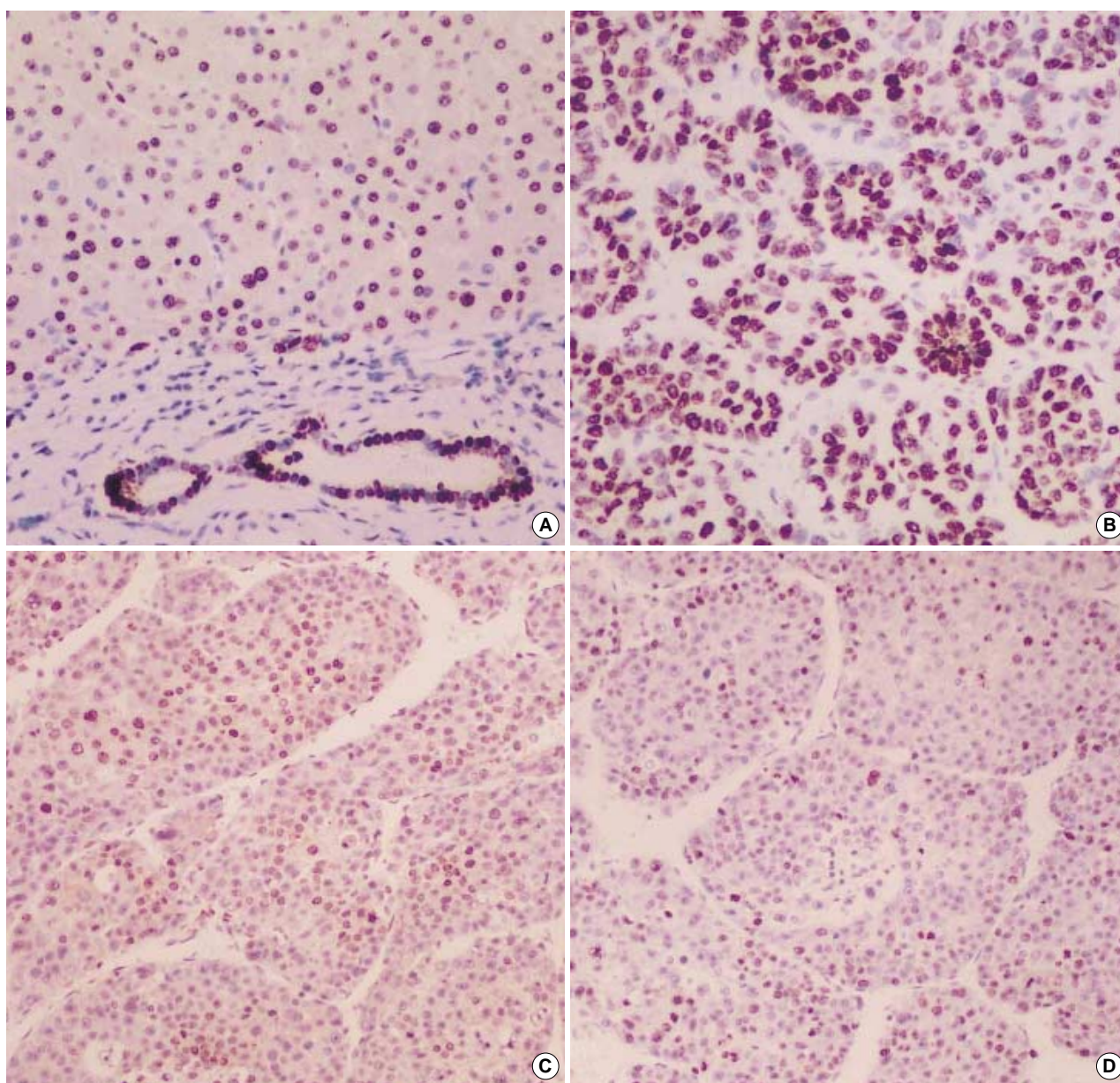


Fig. 2. Expression of Cyclin D1 protein (A, B, and C) and Ki-67 immunostaining (D). (A) Nonneoplastic hepatocytes showing scattered cyclin D1-positive nuclei. (B) Well-differentiated HCCs showing high cyclin D1 labeling index, and (C) Localization of cyclin D1-positive cells to central portion of trabecular or pseudoglandular pattern, discordant to distribution of Ki-67-positive cells (D).

clear overexpression, and no overexpression were $3.2 (\pm 3.0)$, $2.7 (\pm 2.5)$, and $1.7 (\pm 1.4)$, respectively, and there was a significant difference between them ($p=0.049$). HCCs with nuclear expression and nonnuclear overexpression of β -catenin showed a significantly higher mitotic index than cases with no overexpression ($p=0.018$ and 0.038 , respectively). The average MIs of cases showing nuclear expression were slightly higher than those of cases showing nonnuclear overexpression, but there was no statistical significance. No significant difference was found in the average cyclin D1 LIs according to the β -catenin expression patterns.

DISCUSSION

β -Catenin plays a fundamental role in the regulation of the E-cadherin-catenin cell adhesion complex as well as in the Wnt signaling pathway (1-5). The expression of β -catenin in cells is controlled by a multiprotein complex, and mutations in the glycogen synthase kinase 3β (GSK- 3β) phosphorylation sites of the β -catenin gene (CTNNB1) result in nuclear and/or cytoplasmic accumulation of β -catenin and constitutive transactivation of TCF/LEF target genes, a mechanism occurring in many cancers (3-8). The significances of subcellular localiza-

Table 1. Relationship between β -catenin expression patterns and pathologic parameters

	n=77	Altered expression			Nonnuclear overexpression			Nuclear expression		
		present n=45	absent n=32	p-value	present n=29	absent n=48	p-value	present n=16	absent n=61	p-value
Etiology of underlying liver disease										
HBV (+)	73	44	29	NS	28	45	NS	16	57	NS
HCV (+)	4	1	3		1	3		0	4	
Tumor size										
Small (<3 cm)	32	14	18	0.027	8	24	0.044	6	26	NS
Large (\geq 3 cm)	45	31	14		21	24		10	35	
Portal vein invasion										
Absent	31	14	17	NS	6	25	0.006	8	23	NS
Present	46	31	15		23	23		8	38	
Histologic grade										
Well	37	17	20	0.032	10	27	NS	7	30	NS
Poor	40	28	12		19	21		9	31	
Stage										
T1	7	1	6	0.028	1	6	NS	0	7	NS
T2	27	16	11		8	19		8	19	
T3	24	13	11		9	15		4	20	
T4	19	15	4		11	8		4	15	

HBV: HBsAg positive; HCV: anti-HCV positive; Well: Edmondson-Steiner grade I+II; Poor: Edmondson-Steiner grade III+IV; NS: not significant.

Table 2. Relationships of mitotic index and cyclin D1 labeling index with pathologic parameters

Variables	Average mitotic index	p-value	Average cyclin D1 LI	p-value
Tumor size				
Small (<3 cm)	1.43 \pm 1.82	0.002	20.59 \pm 15.35	0.020
Large (\geq 3 cm)	3.07 \pm 2.37		30.24 \pm 19.07	
Portal vein invasion				
Absent	1.06 \pm 1.22	<0.001	27.64 \pm 21.67	NS
Present	3.28 \pm 2.43		25.28 \pm 15.53	
Histologic grade				
Well	1.61 \pm 2.16	0.004	28.32 \pm 17.57	NS
Poor	3.11 \pm 2.21		24.3 \pm 18.69	
Stage				
T1	0.33 \pm 0.43	<0.001	32.86 \pm 15.77	NS
T2	1.2 \pm 1.19		26.37 \pm 22.12	
T3	2.43 \pm 1.14		26.33 \pm 13.80	
T4	4.77 \pm 2.98		23.47 \pm 18.25	

Well: Edmondson-Steiner grade I+II; Poor: Edmondson-Steiner grade III+IV; NS: not significant.

Table 3. Relationships of mitotic index and cyclin D1 labeling index with β -catenin expression

β -catenin expression patterns	Average MI	p-value	Average cyclin D1 LI	p-value
No overexpression	1.66 \pm 1.37	0.038	26.72 \pm 14.4	NS
Nuclear expression	3.21 \pm 3.03		22.44 \pm 22.4	
Nonnuclear overexpression	2.74 \pm 2.5		27.79 \pm 19.6	

NS: not significant.

tion of β -catenin using immunohistochemistry have been reported to be variable according to the tumor types. In colorectal cancer, increased cytoplasmic and nuclear staining seems to be an independent predictor of poor survival (28), whereas decrease or loss of expression correlated with poor prognosis in gastric or pancreatic adenocarcinoma (29, 30). To date, the

reported mutations of β -catenin in HCC involve predominantly GSK-3 β phosphorylation sites in exon 3, suggesting inappropriate activation of the Wnt signaling pathway, and a high association between the nuclear β -catenin expression and gene mutation was previously shown (9-16).

Most of the previous studies had focused on the nuclear ex-

pression pattern of β -catenin (11, 12, 14, 15). There are several conflicting reports concerning the association of β -catenin with tumor progression and patient's survival: the results published by Hsu et al. (12) and Mao et al. (13) demonstrated that expression of mutant nuclear β -catenin correlated with low stage of HCC and favorable prognosis. However, according to the results of some recent studies (15, 16), nuclear expression seems to correlate with tumor progression and poor prognosis. In our study, altered expressions of β -catenin including nonnuclear overexpression and nuclear expression were detected in 58.4% of HCC and showed significant correlations with large tumor size, poor histologic grade, and high tumor stage. However, we are not able to find any relationship between nuclear expression of β -catenin and pathologic features of HCCs. In contrast, nonnuclear overexpression out of two altered expression patterns was more frequent (37.7% versus 20.8%) as well as pathologically more significant than nuclear expression. With regard to proliferative activity, not only nuclear expression but also nonnuclear overexpression of β -catenin correlated significantly with high mitotic index. Nonnuclear overexpression of β -catenin had been previously mentioned by Endo et al. (31), Nhieu et al. (15), Wong et al. (11), and Wei et al. (32). Especially, Wong et al. (11) stressed that the nonnuclear type β -catenin overexpression correlated significantly with poor prognostic factors in predominantly HBV-associated HCCs as noted in our study, however, they did not suggest any possible explanation. According to our results, nonnuclear overexpression of β -catenin appeared to be a frequent finding in predominantly HBV-associated HCC and can contribute tumor progression by stimulating tumor cell proliferation.

Previous studies have provided evidence that nuclear expression of β -catenin stimulates tumor cell proliferation or tumor progression (15), however, little is known about the effect of cytoplasmic β -catenin. Although molecular mechanism of nonnuclear overexpression pattern remains elusive, it is possibly different from that of the nuclear expression pattern due to the following reasons. First, it is generally accepted that subcellular distribution of β -catenin regulates its function; membrane-bound β -catenin predominantly mediates cell-cell adhesion, whereas elevation of the cytoplasmic and nuclear pool of the protein is associated with an oncogenic function. Nonnuclear overexpression pattern noted in our study was mainly membranous staining. Second, in our study, HCCs with nonnuclear overexpression showed a high association with portal vein invasion ($p=0.006$), suggesting that this phenomenon might be an effect related to alteration of cell-cell adhesion facilitating vascular invasion. Wei et al. (32) also reported that vascular invasion was frequently noted in HCCs showing enforced expression of the membranous E-cadherin/ β -catenin complex, and they proposed that dynamic up- and down-regulations of these cell adhesion molecules might be required for the malignant progression of HCC. Third, most of the previous studies had focused on the nuclear expression

pattern and showed a high association between the nuclear expression of β -catenin and HCV-related HCCs (11, 12, 14-16), whereas HCCs examined in the present study and by Wong et al. (11) were predominantly HBV-associated, in which nonnuclear overexpression pattern was predominant and showed significant correlations with pathologic parameters. Although these facts are not sufficient to suggest a close relationship between nonnuclear overexpression and HBV-related HCCs, nonnuclear overexpression might be frequently associated with HBV-related HCCs. Further investigation is necessary to resolve this issue.

Although the exact target genes of β -catenin/TCF4 are still unknown, cyclin D1, one of the major cell cycle regulators, has been reported as a possible target (19, 20). In our study, we failed to find an association between the expressions of β -catenin and the cyclin D1. Considering the reports that overexpression of an oncogenic form of β -catenin in the liver did not induce an up-regulation of cyclin D1 in transgenic mice (33), there might be another pathway or target genes at work in hepatocarcinogenesis.

Cyclin D1 function is generally presumed to be associated with cell proliferation. However, contrary to our expectations, the results in our study showed no correlation between cyclin D1 expression and the mitotic index as well as different topologic distribution between cyclin D1 and Ki-67 in some cases. Meanwhile, cyclin D1 LI showed a significant correlation to large tumor size, which was partially in line with the previous reports showing that cyclin D1 overexpression exhibits an advanced clinicopathologic appearance (22, 23). Based on our results, we could reasonably infer that cyclin D1 expression may be unrelated to active cell proliferation, and may confer additional growth advantages to the tumor progression.

In conclusion, our results indicate that the altered expression of β -catenin in HCC may play an important role in tumor progression by stimulating tumor cell proliferation, and nonnuclear overexpression may have pathologic significance.

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