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β-Catenin Accumulation and Mutation of Exon 3 of the *β-Catenin* Gene in Hepatocellular Carcinoma

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A study was conducted to clarify the contribution of β-catenin accumulation and mutation of the *β-catenin* gene to hepatocarcinogenesis. β-Catenin accumulation was examined immunohistochemically in 38 paired samples of hepatocellular carcinoma (HCC) and corresponding non-cancerous liver tissue. Gene mutation was analyzed by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) and direct sequencing using intronic primers encompassing exon 3. Neither accumulation nor mutation was detected in non-cancerous liver tissues that showed no remarkable histological features, chronic hepatitis or liver cirrhosis. Accumulation of β-catenin was seen in the nucleus, cytoplasm or cell membrane in 15 of 38 (39%) HCC samples, and gene mutation was seen in 9 of 38 (24%) HCC samples. Although there was a significant correlation between accumulation and mutation ($P<0.01$), six HCCs without mutation also showed accumulation. Samples of early HCC showed neither accumulation nor mutation, and accumulation and mutation were each correlated significantly with portal vein tumor involvement ($P<0.05$). The present results indicate that (1) mutation of exon 3 of the *β-catenin* gene can lead to β-catenin accumulation, although other mechanisms of accumulation may also operate in HCC, and (2) β-catenin accumulation and mutation of the *β-catenin* gene are not early events in hepatocarcinogenesis, and may be associated with the malignant progression of HCC.

Key words: Hepatocellular carcinoma — β-Catenin — Single strand conformation polymorphism

β-Catenin is one of the undercoat proteins of cadherin cell adhesion molecules.[1, 2)] This protein plays a role in the cross-talk between the wingless/Wnt signal transduction pathway, which is essential for embryonic development and tissue organization, and the cadherin-mediated cell adhesion system.[3–6)] The intracellular level of β-catenin is regulated by proteasomal degradation through complex formation with adenomatous polyposis coli (APC) protein and by phosphorylation (by glycogen synthase kinase-3β; GSK-3β) of the serine/threonine residues encoded by exon 3 of the *β-catenin* gene.[5, 6)] In some human cancers, mutation of either the *APC* gene or the *β-catenin* gene itself, especially in exon 3, leads to the accumulation of β-catenin in the cancer cells.[7–9)] The aberrantly accumulated β-catenin binds to members of the T cell-factor/lymphoid-enhancer-factor (Tcf/Lef) protein family and the complex acts as a transcriptional regulator.[7, 10)]

Two investigations of mutations of the *β-catenin* gene in hepatocellular carcinoma (HCC) have been reported recently. However, they did not include immunohistochemical examinations of primary HCCs and did not examine the association between β-catenin accumulation and clinicopathological parameters.[11, 12)] Therefore, the contribution of β-catenin to hepatocarcinogenesis is not well understood. We have performed mutation analysis of the *β-catenin* gene and have detected β-catenin immunohistochemically in primary HCCs and corresponding non-cancerous liver tissues. We examined the relationships between aberrations of β-catenin and the clinicopathological features of HCC.

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MATERIALS AND METHODS

Tissue samples Paired samples of cancerous tissue and non-cancerous liver tissue were obtained from 34 HCC patients who underwent therapeutic surgical resection at the National Cancer Center Hospital, Tokyo (Table I). One tumor ($13T_1$) was classified as early HCC, in which the pre-existing liver structure is well preserved and is considered to correspond to *in-situ* or microinvasive HCC.[13, 14)] $13T_1$ was grade I and, 2, 18, 14 and 1 advanced HCCs were grade I, II, III and IV, according to Edmondson's classification.[15)] Patient 9 had a nodule-in-nodule-type HCC that was composed of a progressed HCC component ($9T_2$) surrounded by an extensive area of early HCC component ($9T_1$). Patient 13 had two HCCs ($13T_1$ and $13T_2$) in separate segments of the liver. Patient 21 had two HCCs ($21T_1/T_2$ and $21T_3$). $21T_1/T_2$ was a nodule-in-nodule-type HCC; a progressed HCC component ($21T_2$) was sur-

Table I. Background of Patients with Hepatocellular Carcinomas

Case	Age	Sex	Tumor				Non-cancerous[c] liver tissue	Viral status[d]
			Histological[a] grade	Size (cm)[b]	Involvement of portal vein	Intrahepatic metastasis		
1	71	F	II	3.8	–	–	LC	C
2	71	M	III	2.5	–	–	CH	NBNC
3	54	F	II	6	+	+	CH	NBNC
4	77	M	III	5	+	–	CH	C
5	65	M	III	5	+	–	CH	C
6	58	M	III	5	–	–	CH	NBNC
7	66	M	II	11	+	+	N	NBNC
8	48	M	II	9	+	+	LC	C
9	48	M	I (T_1/T_2)	9.5	–	–	CH	NBNC
10	67	M	II	4	+	–	CH	C
11	66	M	III	11	+	–	LC	C
12	20	M	III	3	+	+	N	B
13	48	M	I (T_1, early)	0.7	–	–	LC	B
			III (T_2)	7.5	+	+		
14	67	F	II	4	+	+	LC	C
15	64	M	II	1.3	–	–	CH	C
16	61	M	IV	13	+	–	CH	B
17	74	M	III	1.7	–	–	LC	C
18	53	F	II	13	+	+	CH	B
19	56	M	II	2	+	–	CH	C
20	76	M	II	4.5	+	–	N	NBNC
21	65	M	I (T_1/T_2)	1.8	–	–	LC	C
			III (T_3)	9	+	–		
22	71	F	II	2	+	–	LC	C
23	75	M	II	3	–	–	LC	C
24	40	M	III	3	–	–	LC	NBNC
25	65	M	II	4.5	+	–	LC	C
26	59	M	II	13.8	–	–	LC	C
27	47	M	II	1.8	–	–	CH	B
28	75	M	III	11	–	–	N	NBNC
29	59	M	II	4.5	+	–	LC	B&C
30	77	M	II	6	+	+	LC	C
31	64	M	III	9	+	+	CH	C
32	65	M	II	11.3	+	–	CH	B
33	62	M	III	2	+	–	CH	C
34	57	M	III	4.5	+	+	CH	C

a) According to Edmondson's classification. Case 9 had a nodule-in-nodule-type HCC (size; 9.5 cm): progressed HCC component ($9T_2$) was surrounded by early HCC component ($9T_1$). Case 13 had two HCCs ($13T_1$ and $13T_2$) in segments separate from each other in the liver. $21T_1$ and $21T_2$ composed a nodule-in-nodule-type HCC (size; 1.8 cm): progressed HCC component ($21T_2$) was surrounded by early HCC component ($21T_1$). $21T_3$ was in a separate segment from $21T_1/T_2$ in the liver.
b) Represented as the largest diameter.
c) N, histologically normal; CH, chronic hepatitis; LC, liver cirrhosis.
d) B, hepatitis B virus surface antigen (HBs-Ag) positive; C, anti-hepatitis C virus antibody (Anti-HCV) positive; NBNC, HBs-Ag and Anti-HCV negative; B&C, both HBs-Ag and Anti-HCV positive.

rounded by a smaller area of early HCC component ($21T_1$). $21T_3$ and $21T_1/T_2$ were in separate segments of the liver. Data were also available regarding the presence or absence of tumor involvement of the portal vein and intrahepatic metastasis. Hepatitis B virus surface antigen (HBs-Ag) and anti-hepatitis C virus antibody (anti-HCV) were measured.

Immunohistochemistry Immunohistochemical examination was performed as described previously.[16, 17] Briefly, after deparaffinization and rehydration of 5-μm-thick sec-

tions of methanol-fixed paraffin-embedded tissue samples, endogenous peroxidase activity was blocked with 0.3% H_2O_2 and non-specific reaction was blocked with normal swine serum (DAKO, Glostrup, Denmark). The sections were incubated overnight with an anti-β-catenin monoclonal antibody (diluted 1:250; Transduction Laboratories, Lexington, KY) at 4°C. Then the sections were incubated for 30 min at room temperature with biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA) and were treated with Vectastain Elite ABC reagent (Vector Laboratories). 3,3′-Diaminobenzidine tetrahydrochloride was used as the chromogen. All sections were counterstained with hematoxylin. The bile duct epithelium served as a positive control in all sections. For negative control preparations, the primary antibody was omitted from the reaction sequence.

DNA preparation Genomic DNA was extracted and purified from 38 fresh paired samples of cancerous tissue and non-cancerous liver tissue by the phenol-chloroform method.[18] In addition, in 26 cases, we used five lobules per case dissected from non-cancerous liver tissues that showed no remarkable findings or five pseudolobules or regenerative nodules per case dissected from non-cancerous liver tissues that showed chronic hepatitis or liver cirrhosis. Dissection was performed using an 18-guage needle and a stereoscopic microscope.

After immunohistochemical examination of the tissue sections, cells that showed accumulation of β-catenin were dissected using a PixCell laser capture microscope (LCM) with an infrared diode laser (Arcturus Engineering, Santa Clara, CA), as described previously.[19] In brief, sections were overlaid with a thermoplastic membrane mounted on optically transparent caps, and cells were captured by focal melting of the membrane using a laser. After visual confirmation of the completeness of the dissection, the captured cells were immersed in a denaturing solution and the DNA was extracted.

Polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) and direct sequencing Genomic DNA was amplified by PCR using a previously described pair of intronic primers encompassing exon 3 of the *β-catenin* gene, G-F (5′-CCAATCTACTAATGCTAATACTG-3′) and G-R (5′-CTGCATTCTGACTTTCAGTAAGG-3′).[9] PCR amplification was performed using 10 ng of genomic DNA in the presence of [^{32}P-α]dCTP and EX *Taq* polymerase (Takara, Ohtsu) under the following conditions: 2 min at 95°C for initial denaturing followed by 40 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 45 s. The PCR product was diluted in loading buffer (95% formamide, 20 m*M* EDTA, 0.1% bromophenol blue, 0.1% xylene cyanole) and electrophoresed on a 6% polyacrylamide gel at 4°C. The mobility-shifted bands were dissected from the gel and the DNA was recovered and amplified by repeating the PCR using the same primers. The re-amplified PCR product was sequenced using a commercial kit (BigDye Terminator Cycle Sequencing Ready Reaction Kit; Perkin-Elmer, Foster City, CA) and an automated sequencer (model 377; Perkin-Elmer) according to the manufacturer's protocol. Sequencing in both directions was performed using the PCR primers.

PCR amplification encompassing exon 3 To examine large-scale abnormalities involving all or a part of exon 3, genomic DNA was amplified by PCR using a pair of primers that corresponded to the sequences of exons 2 and 4 of the *β-catenin* gene, C-F (5′-CCAGCGTGGACAATGGCTAC-3′) and C-R (5′-TGAGCTCGAGTCATTGCATAC-3′), respectively.[9] The amplified product was analyzed by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide, and examined under ultraviolet light.

Statistical analysis The χ^2 test, Fisher's test and Student's *t* test were used for statistical analyses. *P* values of less than 0.05 were considered to indicate significance.

RESULTS

Accumulation of β-catenin Non-cancerous hepatocytes in both liver tissues that showed no remarkable histological findings and liver tissues that showed chronic hepatitis or liver cirrhosis lacked β-catenin immunoreactivity in the nucleus and cytoplasm, and showed only weak immunoreactivity on the cell membrane (Fig. 1A). By contrast, some cancer cells showed prominent β-catenin immunoreactivity in the nucleus, cytoplasm or cell membrane (Fig. 1, B–D, Table II). Nuclear β-catenin immunoreactivity was heterogeneous; less than 50% of the cancer cells of each HCC examined showed nuclear staining (Fig. 1C, Table II).

Accumulation of β-catenin, compared to the amounts seen in non-cancerous hepatocytes, was detected in 15 of 38 (39%) HCC samples; β-catenin accumulation in both the cytoplasm and membrane was seen in all 15 HCCs and 4 of them also showed protein accumulation in the nucleus (Table II). One early HCC ($13T_1$) and early HCC component ($9T_1$ and $21T_1$) of two nodule-in-nodule-type HCCs that we examined showed no β-catenin accumulation. Indeed, in the nodule-in-nodule-type HCC of case 21 there was a clear distinction between the progressed HCC component ($21T_2$) and the early HCC component ($21T_1$); β-catenin accumulation in the nucleus, cytoplasm and cell membrane was seen in $21T_2$, whereas no accumulation was seen in $21T_1$ (Fig. 1D).

β-Catenin accumulation in HCCs correlated significantly with portal vein tumor involvement ($P<0.05$, Table III). There was no significant correlation between β-catenin accumulation and other clinicopathological parameters, e.g. status of the non-cancerous liver tissues (histologically normal, chronic hepatitis or liver cirrhosis) and viral status (Table III).

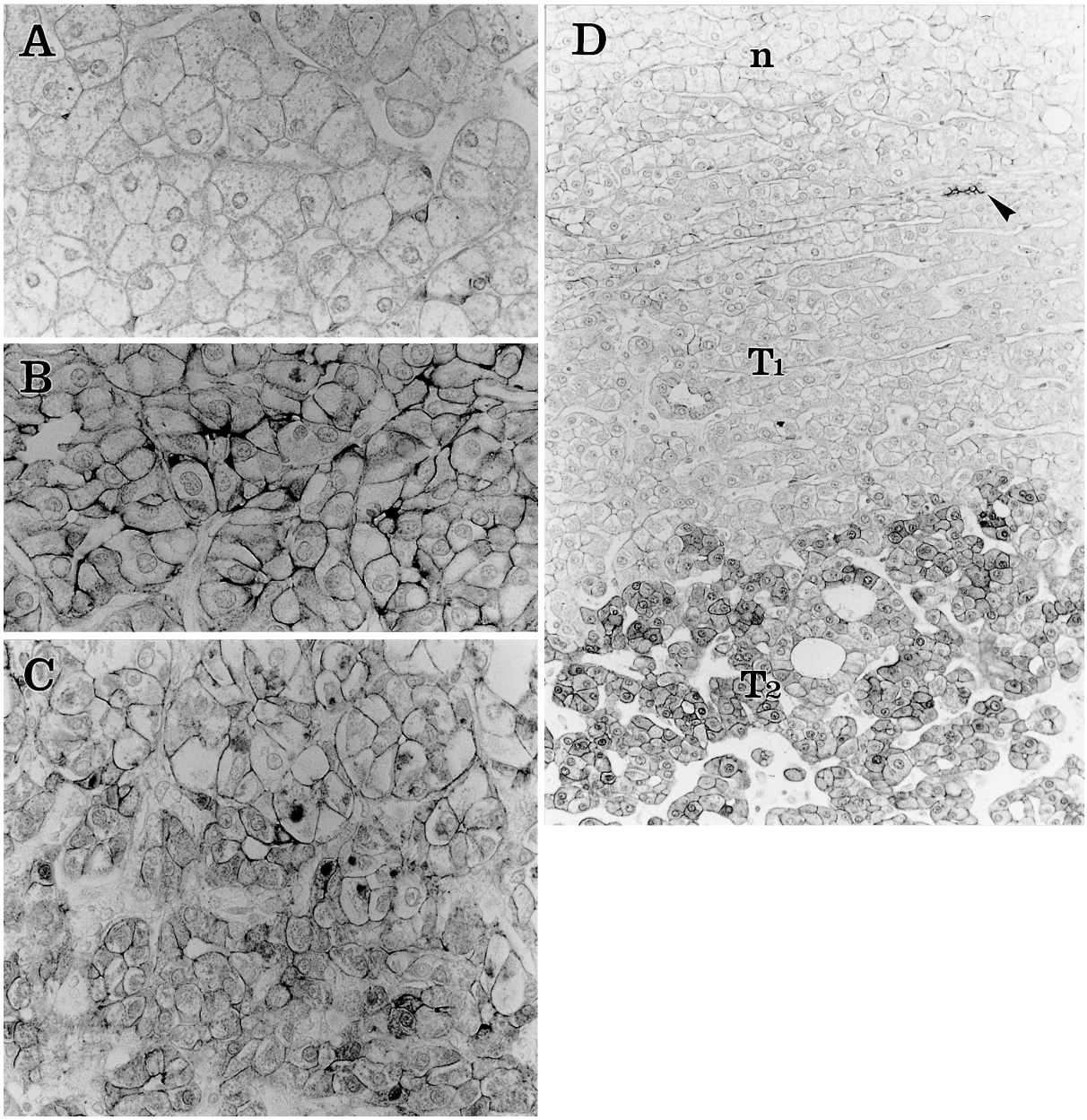

Fig. 1. Immunohistochemical examination of β-catenin. Bile duct epithelium served as the positive control (arrowhead). (A) In all cases, non-cancerous hepatocytes did not show immunoreactivity in the nucleus or cytoplasm, but showed weak immunoreactivity on the cell membrane. (Representative section, case 32; ×350.) (B) In case 12, the cancer cells showed prominent immunoreactivity in the cytoplasm and on the cell membrane (×350). (C) In case 20, the cancer cells showed prominent immunoreactivity in nucleus, cytoplasm and cell membrane. Nuclear staining was heterogeneous among cells in the same section; about 10% of the cancer cells were positive in this case (×350). (D) In case 21 the tumor was a nodule-in-nodule-type HCC. Cells of the progressed HCC component (T_2) showed immunoreactivity on nucleus, cytoplasm and cell membrane, whereas the cells of the surrounding early HCC component (T_1) and the non-cancerous hepatocytes (n) lacked prominent immunoreactivity (×180).

Mutations in exon 3 of the *β-catenin* gene PCR-SSCP analysis revealed no mobility shift of the *β-catenin* gene DNA in the non-microdissected non-cancerous liver tissues or in the dissected non-cancerous lobules, pseudolobules and regenerative nodules. By contrast, a mobility-shifted band was detected in 9 of 38 (24%) HCC samples

Table II. Results of Immunohistochemical Examination and Mutation Analysis of β-Catenin in Hepatocellular Carcinoma Samples

Tumor samples	Immunohistochemistry			Mutation analysis		
	Nucleus[a)]	Cytoplasm[b)]	Cell membrane[c)]	SSCP[d)]	Affected codon	Mutation
1	−	−	+	−		
2	−	−	+	−		
3	−	−	+	−		
4	−	+	2+	+	34	GGA (Gly)→GAA (Glu)
5	−	−	+	−		
6	−	−	+	−		
7	−	−	+	−		
8	−	+	2+	+	37	TCT (Ser)→TTT (Phe)
$9T_1$	−	−	+	−		
$9T_2$	−	−	+	−		
10	−	−	+	−		
11	−	+	2+	+	45	TCT (Ser)→TGT (Cys)
12	−	+	2+	−		
$13T_1$	−	−	+	−		
$13T_2$	−	+	2+	−		
14	−	−	+	−		
15	−	−	+	−		
16	−	−	+	−		
17	−	−	+	−		
18	−	+	2+	−		
19	−	−	+	−		
20	+	+	2+	+	35	ATC (Ile)→AGC (Ser)
$21T_1$	−	−	+	−		
$21T_2$	±	+	2+	+	32	GAC (Asp)→GCC (Ala)
$21T_3$	±	+	2+	+	32−48	51-bp deletion
22	−	+	2+	+	41	ACC (Thr)→GCC (Ala)
23	−	+	2+	−		
24	−	−	+	−		
25	±	+	2+	−		
26	−	−	+	−		
27	−	−	+	−		
28	−	−	+	−		
29	−	−	+	−		
30	−	+	2+	−		
31	−	−	+	−		
32	−	+	2+	+	35	ATC (Ile)→AGC (Ser)
33	−	−	+	−		
34	−	+	2+	+	32	GAC (Asp)→AAC (Asn)
Total (%)	4 (11)	15 (39)	15 (39)	9 (24)		

a) −, none; ±, less than 5%; +, 5−50% of cancer cells showed immunoreactivity for β-catenin on the nucleus.
b) −, cytoplasmic staining was not observed; +, observed.
c) +, membranous staining was equivalent to that in non-cancerous hepatocytes; 2+, membranous staining was stronger than that in non-cancerous hepatocytes.
d) −, mobility band shift was not observed; +, observed with single strand conformation polymorphism.

(Fig. 2, Table II). Sequence analysis revealed that eight were point mutations and one was an in-frame deletion of a 51-bp nucleotide sequence of the *β-catenin* gene (Fig. 2, Table II). All of the point mutations were missense mutations that lead to the replacement of a serine or threonine residue, encoded by codons 33, 37, 41 and 45, or of residues flanking one of these serine and threonine residues. The deleted 51-bp region in $21T_3$ included codons 32−48.

Table III. Associations between Protein Accumulation and Gene Mutation of β-Catenin and Clinicopathological Parameters of Patients with Hepatocellular Carcinomas

			Number of tumor samples		
			Analyzed	Protein accumulation[f] detected (%)	Gene mutation detected (%)
Histology	early	I[a]	3[b]	0 (0)	0 (0)
	advanced	I	2	1 (50)	1 (50)
		II	18	8 (44)	5 (28)
		III	14	6 (43)	3 (21)
		IV	1	0 (0)	0 (0)
Size[c]		3.0 cm>	13	4 (31)	2 (15)
		3.0 cm≤	25	11 (44)	7 (28)
Involvement of portal vein		(–)	15	1 (7)[g]	1 (7)[g]
		(+)	23	14 (61)	8 (35)
Intrahepatic metastasis		(–)	28	9 (32)	6 (21)
		(+)	10	6 (60)	3 (30)
Non-cancerous liver tissue[d]		N	4	2 (50)	1 (25)
		CH	17	4 (24)	3 (18)
		LC	17	9 (53)	5 (29)
Viral status[e]		HBV	7	4 (58)	1 (14)
		HCV	21	10 (48)	7 (33)
		HBV&HCV	1	0 (0)	0 (0)
		NBNC	9	1 (11)	1 (11)
Total			38	15 (39)	9 (24)

a) According to Edmondson's classification.
b) One early HCC ($13T_1$) and early HCC component ($9T_1$ and $21T_1$) of two nodule-in-nodule-type HCCs.
c) Largest diameter.
d) N, histologically normal; CH, chronic hepatitis; LC, liver cirrhosis.
e) HBV, hepatitis B virus surface antigen (HBs-Ag) positive; HCV, anti-hepatitis C virus antibody (Anti-HCV) positive; HBV&HCV, both HBs-Ag and Anti-HCV positive; NBNC, HBs-Ag and Anti-HCV negative.
f) β-Catenin accumulated in the nucleus, cytoplasm and/or cell membrane.
g) The incidences of protein accumulation and gene mutation of β-catenin each correlated significantly with portal vein tumor involvement ($P<0.05$).

One early HCC ($13T_1$) and early HCC component ($9T_1$ and $21T_1$) of two nodule-in-nodule-type HCCs that we examined lacked a gene mutation in exon 3.

To prove the relationship between β-catenin accumulation and mutation of the *β-catenin* gene, cancer cells that showed β-catenin accumulation were dissected from immunostained sections of 15 HCCs using the LCM system. PCR-SSCP and direct sequencing revealed that cancer cells with β-catenin accumulation from nine HCCs had a mutation in exon 3 and that those from the other six HCCs did not. These results were consistent with our PCR-SSCP results and the direct sequencing data for the non-microdissected cancerous tissues. In case 21, LCM microdissection of liver tissues and mutation analysis were performed for both the progressed HCC component ($21T_2$), which showed β-catenin accumulation, and for the small early HCC component ($21T_1$), which lacked β-catenin accumulation (Fig. 1D). A mutation was detected in the *β-catenin* gene in $21T_2$, but not in $21T_1$ (Fig. 2B).

In addition, we looked for large-scale abnormalities involving exon 3, which are frequently detected in colorectal cancers,[9] by PCR amplification. The only large-scale abnormality detected was the 51-bp deletion in $21T_3$, which we had already detected by PCR-SSCP and direct sequencing.

Mutation of the *β-catenin* gene in HCCs correlated significantly with portal vein tumor involvement ($P<0.05$, Table III). There was no significant correlation between

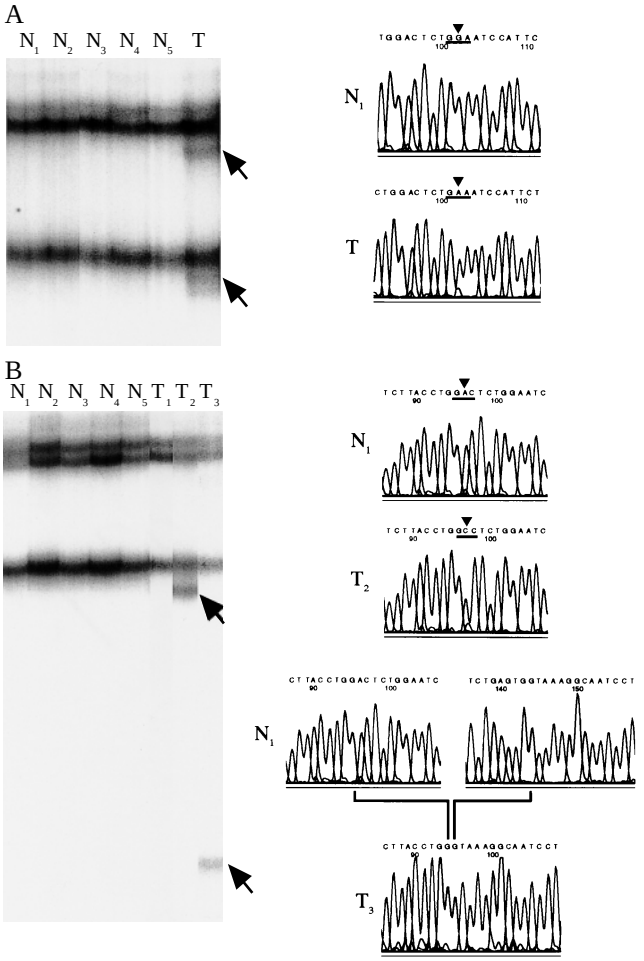

Fig. 2. PCR-SSCP and direct sequencing of the β-catenin gene. (A) In case 4, a mobility-shifted band of DNA (arrows) was detected in the cancerous tissue (T), but not in five microdissected pseudolobules (N_1 to N_5). Direct sequencing of the shifted band revealed a point mutation (G to A, arrowheads) in codon 34 (underlined). (B) In case 21 the tumor was a nodule-in-nodule-type HCC of progressed HCC component (T_2) surrounded by early HCC component (T_1). T_3 was a grade III HCC in a separate liver segment. Mobility-shifted bands of DNA (arrows) were detected in T_2 and T_3, but not in five microdissected regenerative nodules (N_1 to N_5). DNA from cancer cells that were microdissected from immunostained sections of T_1 did not show a mobility shift. Direct sequencing of the shifted bands from T_2 and T_3 revealed a point mutation (T to C, arrowheads) in codon 32 (underlined) and a 51-bp deletion (codons 32 to 48), respectively.

mutation of the *β-catenin* gene and other clinicopathological parameters, e.g. status of non-cancerous liver tissues (histologically normal, chronic hepatitis or liver cirrhosis) and viral status (Table III).

The significant correlation between β-catenin accumulation and mutation of the *β-catenin* gene is summarized in Table IV ($P<0.01$). All of the HCCs with a *β-catenin* gene mutation in exon 3 showed β-catenin accumulation, though six HCCs without a *β-catenin* gene mutation in exon 3 also showed β-catenin accumulation.

Table IV. Associations between Protein Accumulation and Gene Mutation of β-Catenin in Hepatocellular Carcinoma Samples

		Protein accumulation[a)]		*P*
		+	–	
Gene mutation[b)]	+	9	0	0.007
	–	6	23	

a) +, β-Catenin was accumulated in the nucleus, cytoplasm and/or cell membrane; –, not accumulated.
b) +, Mutation of the *β-catenin* gene was detected; –, not detected.

DISCUSSION

β-Catenin that has accumulated in the cytoplasm to high levels binds to Tcf/Lef proteins and influences transcription through the DNA-binding domain of the Tcf/Lef proteins and perhaps through a potential transactivation domain of β-catenin itself.[7, 10] Recently, various target genes of the β-catenin-Tcf/Lef complex (c-myc, AP-1, fra-1, c-jun, urokinase-type plasminogen activator receptor and cyclin D1) were reported to show altered expression in colorectal cancers.[20–22] Transcriptional aberrations of these genes may participate in human carcinogenesis. In this study, immunohistochemical examination revealed a high incidence of β-catenin accumulation in primary HCCs, suggesting that β-catenin accumulation may play a role in hepatocarcinogenesis.

In addition to β-catenin accumulation in the nucleus and cytoplasm, which is common in colorectal cancers and other human cancers, we observed prominent immunoreactivity for β-catenin on the cell membrane in HCCs. Since non-cancerous hepatocytes showed weak β-catenin immunoreactivity, they may be able to capture excess β-catenin molecules in the membrane-spanning cadherin-catenin cell adhesion system, that is, the high levels of β-catenin on the cell membranes of HCCs may be due to the capacity of the cadherin system to bind mutant β-catenin molecules.

The intracellular level of β-catenin is regulated by proteasomal degradation, which requires the phosphorylation by GSK-3β of target serine/threonine residues encoded in exon 3 of the *β-catenin* gene.[4, 5] In the present study, nine (24%) HCCs showed either a point mutation or a deletion that involved one or more phosphorylation target residues or their flanking residues, which are thought to affect the

protein conformation around the target residues. Indeed, all of the HCCs with a *β-catenin* gene mutation in exon 3 showed β-catenin accumulation; β-catenin accumulation and mutation of exon 3 of the *β-catenin* gene correlated significantly ($P<0.01$). The incidence of *β-catenin* gene mutation observed in the present study is similar to figures reported in previous studies, although they did not perform immunohistochemical examination in the same primary HCCs.[11, 12] This report is the first to show that mutation in exon 3 of the *β-catenin* gene correlates with β-catenin accumulation in HCCs.

On the other hand, six HCCs without *β-catenin* gene mutations in exon 3 also showed β-catenin accumulation. Although it cannot be ruled out that mutations of the *β-catenin* gene in exons other than exon 3 resulted in β-catenin accumulation in these 6 cases, such mutations have been reported in only a few human cancers.[23] Mutations of the *APC* gene can cause stabilization and accumulation of β-catenin in colorectal cancers,[7, 9] but such mutations are probably very rare in HCCs.[24, 25] Therefore, mechanisms other than mutations of the *β-catenin* or *APC* genes may cause β-catenin accumulation in HCCs. Dysfunction of axin, which forms a complex with GSK-3β and participates in phosphorylation of β-catenin,[26] is a candidate mechanism. Further studies are needed to identify alternative mechanisms of β-catenin accumulation in HCCs.

All of the non-cancerous liver tissues, an early HCC and early HCC component of two nodule-in-nodule-type HCCs that we examined showed neither β-catenin accumulation nor mutation of the *β-catenin* gene. Moreover, the nodule-in-nodule-type HCC in case 21 showed β-catenin accumulation and a *β-catenin* gene mutation in the progressed HCC component ($21T_2$), but not in the surrounding early HCC component ($21T_1$; Figs. 1D and 2B). Therefore, β-catenin accumulation and mutation of the *β-catenin* gene are not early events of hepatocarcinogenesis. On the other hand, the incidence of β-catenin accumulation and the incidence of *β-catenin* gene mutation each correlated significantly with portal vein tumor involvement ($P<0.05$), so they may be associated with the progression of HCC.

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