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Short Communication

Nuclear Accumulation of Mutated β-Catenin in Hepatocellular Carcinoma Is Associated with Increased Cell Proliferation

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Inappropriate activation of the Wnt pathway resulting from β-catenin gene alterations has recently been implicated in the development of hepatocellular carcinoma (HCC). To explore the *in vivo* effects of mutated β-catenin, HCC specimens from 32 patients carrying one or several tumors were screened for somatic mutations in exon 3 of the β-catenin gene, and the expression and subcellular localization of β-catenin was studied by immunohistochemistry. Missense mutations or interstitial deletions in β-catenin exon 3 were detected in 12 of 35 (34%) HCC samples. After immunostaining, most tumors exhibited increased membranous and/or cytoplasmic expression of β-catenin compared with adjacent nontumoral liver. Strong nuclear accumulation of β-catenin was observed either focally or uniformly in 15 of 35 (43%) tumor specimens, but not in cirrhotic nodules or dysplastic liver cells in adjacent liver. Aberrant nuclear expression of β-catenin was significantly associated with the presence of mutations in the β-catenin gene ($P < 0.005$). Moreover, nuclear β-catenin staining correlated significantly with increased Ki-67 proliferative index in tumor ($P < 0.001$) and seemed to be associated with poor outcome in patients with HCC. In conclusion, our data indicate that activation of the Wnt/β-catenin pathway in HCC results mainly from somatic mutations in the β-catenin gene and may promote tumor progression by stimulating tumor cell proliferation. *(Am J Pathol 1999, 155:703–710)*

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide and a major cause of death in many countries.[1] Although major environmental risk factors, ie, chronic viral infections and hepatotoxic agents, have been identified, the molecular bases of liver cell transformation remain poorly understood.[2] Consistent with the multifactorial etiology of HCC, allelotype studies showing loss of heterozygosity at multiple chromosomal loci suggest that inactivation of different tumor suppressor genes might be implicated in the tumoral process.[3] Among known cancer genes, genetic alterations have been reported for p53, Rb, and the mannose-6-phosphate/IGF II receptor,[4–6] and it has been shown that the c-*myc* oncogene and cyclin D1 are frequently overexpressed.[7] In this context, the recent finding of frequent oncogenic mutations in the β-catenin gene in human and mouse liver tumors,[8,9] together with the identification of c-*myc* and cyclin D1 as target genes of β-catenin signaling,[10,11] have provided essential steps toward understanding the genesis of liver tumors.

β-catenin, like its homologue armadillo in *Drosophila*, is an important multifunctional protein involved in cell-cell adhesion, by strengthening the linkage of cadherin and α-catenin to the actin cytoskeleton.[12] It is also involved in Wingless/Wnt signaling during embryonic development[13] and inappropriate reactivation of this pathway has been implicated in tumorigenesis. In the absence of Wnt signaling, β-catenin is phosphorylated at N-terminal serine-threonine residues by functional interactions with glycogen synthase kinase (GSK)−3β, axin and the adenomatous polyposis coli protein (APC), and subsequently targeted to degradation by the ubiquitin-proteasome system.[14] Activation of the Wnt signal inhibits GSK-3β activity and induces β-catenin stabilization. Translocation of β-catenin to the nucleus and its association with high mobility group domain factors Tcf/LEF causes transcriptional activation of target genes.[15]

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Lately, the c-*myc*, cyclin D1, and WISP genes have been reported to be directly or indirectly activated by Wnt/β-catenin signaling in human epithelial cells.[10,11,16]

Recently, mutant β-catenins that are resistant to down-regulation by GSK-3β phosphorylation and ubiquitination have been characterized in human colorectal cancers and in a variety of carcinomas.[17–19] In colon cancers, immunohistochemical studies have demonstrated increased expression of β-catenin and its nuclear localization in tumors harboring either APC defects or β-catenin mutations in the GSK-3β phosphorylation domain.[18,20] Nuclear and cytoplasmic localization of β-catenin was also frequently seen in ovarian and uterine carcinomas and in melanomas, although genetic alterations of the β-catenin gene could be detected only in a minority of these tumors.[21–23] Whether inactivation of APC or other genetic or epigenetic event contributed to activating the Wnt/β-catenin pathway in these cancers remains to be determined.

In human HCC, mutations of the β-catenin gene have been reported in 19 to 26% of primary tumors,[8,9] but immunolocalization of β-catenin in tumoral tissues has not been investigated. The purpose of the present work was to determine the expression level and subcellular localization of β-catenin in primary HCC specimens and in adjacent livers, to assess the relationship between nuclear accumulation and presence of β-catenin mutations, and to investigate the effects of β-catenin activation on cell proliferation. The expression of β-catenin was also evaluated with respect to tumor size and grade and to patients' clinical follow-up.

Materials and Methods

Patients and Tissue Material

Surgical liver resections for HCC from 32 patients (29 men, 3 women, mean age 59 years, range 36–78 years) were retrospectively studied. HCC was associated with cirrhosis or chronic hepatitis due to HCV (11 cases) or HBV infection (5 cases), to alcohol alone (9 cases) or in association with HCV infection (2 cases), or to genetic hemochromatosis (2 cases). Etiology was undetermined in one case. Two HCCs developed on normal liver. Twenty-two patients underwent segmental hepatectomy and 10 patients underwent orthotopic liver transplantation.

Fresh tissue samples from tumoral nodules and nontumoral livers were snap-frozen in liquid nitrogen and stored at −80°C for DNA studies.

For histopathological and immunohistochemical studies, tumor and liver specimens were fixed in 10% formalin, then embedded in paraffin. Four-micron sections were stained with hematoxylin-eosin-safran and with Masson's trichrome or picrosirius red for collagen. Tumor size, number of tumoral nodules, and presence of vascular invasion were determined at gross and/or microscopic examination.

DNA Analysis

DNA was extracted and purified by standard techniques from frozen tumoral and nontumoral liver samples.[3] Genomic DNAs were amplified by a step-down polymerase chain reaction (PCR) protocol using 100 ng of template DNA, the forward primer 5′-GCGTGGACAATGGCTACTCAAG-3′ (3′ end of exon 2), and the reverse primer 5′-CTGGTCCTCGTCATTTAGC-3′ (3′ end of exon 4). PCR was performed in 50 μl of PCR buffer (Eurobio, Les Ulis, France) with 1.5 mmol/L MgCl2, 200 μmol/L dNTP, 1 μmol/L each primer, and 1 unit of *Taq* polymerase (Eurobio). The PCR conditions consisted of 1 cycle at 94°C for 4 minutes; 3 cycles of denaturation (94°C, 45 seconds), annealing (68°C, 45 seconds), and extension (72°C, 45 seconds); 3 cycles with annealing steps at 65, 62, 59, and 56°C; 20 cycles with annealing step at 52°C, and a final extension cycle at 72°C for 5 minutes in a Cyclogene thermal cycler (Techne, Cambridge, UK). Reaction products were resolved in 1% agarose and visualized with ethidium bromide. In two samples, shorter PCR products corresponding to deleted β-catenin were excised from the gel and eluted. PCR products were sequenced using the T7 Sequenase PCR product sequencing kit (Amersham France, Les Ulis, France) according to the manufacturer's instructions. The primers for sequencing were the forward primer 5′-TGATGGAGT TGGACATGGCCATG-3′ and the reverse primer 5′-CCC ACTCATACAGGACTTGGGAGG-3′ in exon 3.

Immunohistochemical Study

Analysis of β-catenin and MIB-1 (Ki-67) expression was performed on serial sections for each tumor. For β-catenin immunostaining, an immunoenzymatic method using alkaline phosphatase-anti-alkaline phosphatase (APAAP) complexes (Dakopatts, Trappes, France) and a β-catenin monoclonal antibody (Transduction Laboratories, Lexington, KY) was used at a dilution of 1:500. For increased sensitivity and better subcellular localization of β-catenin, an antigen retrieval method using microwave oven heating (2 × 5 minutes) in 1 mmol/L EDTA, pH 8.0,[24] was applied on the deparaffinized and rehydrated sections. Slides were immersed in 20% AB human serum in 0.05 mol/L Tris-buffered saline, pH 7.6, and then incubated overnight at 4°C with the primary antibody. After washing, the slides were incubated for 30 minutes with a second rabbit anti-mouse antibody (Dakopatts) diluted 1:20, washed again, and incubated with the APAAP complexes at a 1:50 dilution. A single amplification was performed by adding sequentially the second antibody for 10 minutes and the APAAP complexes for 10 minutes. Sections were incubated with Fast Red salt-TR - Naphtol AX-TR phosphate (Sigma, Saint-Quentin Fallavier, France) in the presence of levamisole to block endogenous activity, then counterstained with aqueous hematoxylin and mounted in Immumount (Shandon, Cergy-Pontoise, France).

For Ki-67 immunostaining, deparaffinized sections were pretreated in 0.01 mol/L citrate buffer, pH 2.5. Im-

munolabeling, with the monoclonal antibody MIB-1 (Immunotech, Marseille, France) diluted 1:50, was performed using the immunohistochemistry automat Ventana NexES (Ventana Medical System, Strasbourg, France). This automat used the avidin-biotin peroxidase complex (ABC) method with 3,3-diaminobenzidine as chromogen and hematoxylin for counterstaining. As a control, no labeling was observed by omitting the primary antibody.

Evaluation of Immunostaining

The immunostaining of tumor samples was evaluated on coded slides on two different occasions. Membranous and intracytoplasmic β-catenin signals were scored independently and graded in comparison with the adjacent nontumoral liver. In tumors showing nuclear expression, the percentage of strongly positive nuclei was determined by counting at least 1000 tumoral cell nuclei in homogeneous areas. For determination of the proliferative index, stromal cells were excluded and MIB-1-positive tumoral cell nuclei were counted on 10 consecutive high power (×400) fields.[25]

Statistical Analysis

Results of quantitative data in immunohistochemical analyses were expressed as mean ± one SE. Statistical differences between groups were tested with the non-parametric Mann-Whitney *U* test and the Fisher exact probability test. Statistical correlations between immunostaining counts were assessed by the Spearman rank correlation test. The significant level was defined as a *P* value <0.05.

Results

Histopathological Study

All tumors were HCCs grade I to III according to the World Health Organization international histological classification.[26] Most tumors were single, although five were multinodular, and tumor sizes ranged from 1.6 to 19 cm (mean, 7.8 cm). Vascular invasion in portal vein and/or hepatic vein branches was observed in nine cases.

Nontumoral liver was normal in three, fibrotic in six, and cirrhotic in 23 cases.

Identification of β-Catenin Mutations

Thirty-five HCC specimens from 32 patients were screened for mutations in the β-catenin gene by PCR amplification of genomic DNA over intron 2 to exon 4 sequences. The wild-type 1.1-kbp PCR product was present in all tumors, and additional, faster migrating bands were detected in two cases (Figure 1A). Subsequent DNA sequencing of these products revealed interstitial deletions of 553 bp in Patient 11 and 595 bp in Patient 12, encompassing in both cases most of exon 3 and 4 sequences of the β-catenin gene (Figure 1B and Table 1). In the remaining tumors, missense mutations affecting β-catenin residues 32, 33, 34, 35, 36, 40, 41, 45, or 47 were detected in 10 patients (Figure 1D and Table 1). It is noteworthy that these changes affected not only all four GSK-3β phosphorylation sites and flanking residues potentially implicated in β-catenin ubiquitination, but also several residues (H36, T40, and S47) that were not found to have mutated in previous studies of various neoplasms.[19] In Patient 2, among four HCC nodules in the explant liver, only one displayed a mutation changing amino acid 36 from a histidine to a proline. Tumors 7 and 9 are also interesting in that they carried more than one point mutation in exon 3. For Patient 7, in addition to a silent mutation at codon 34, mutations changing isoleucine 35 to an asparagine and threonine 40 to an alanine were demonstrated (Figure 1C), and Patient 9 harbored the classical threonine 41-to-alanine mutation together with a change of serine 47 to an arginine.

Alterations in the β-catenin gene were not statistically related to tumor size, HCC histological grading, or vascular invasion (data not shown). In addition, there was no significant correlation with the etiology of liver disease. The finding that two tumors carrying β-catenin deletion mutants developed in normal livers, and that two HCCs associated with genetic hemochromatosis harbored a T41A mutation (see Table 1) might be coincidental, and larger studies are needed to answer this question.

β-Catenin Immunostaining

In nontumoral livers, a thin membranous β-catenin signal delineated the hepatocytes. Strong membranous and pale cytoplasmic staining of bile ductules, as well as milder staining of nerves and endothelial cells, were observed (Figure 2A). Normal hepatocytes or dysplastic cells from cirrhotic nodules or from livers with chronic hepatitis did not display nuclear expression of β-catenin. In 20 of the 35 tumors analyzed (57%), membranous β-catenin immunostaining was markedly increased by comparison with adjacent livers and was frequently associated with intracytoplasmic staining (data not shown). Nuclear staining could be observed in 23 of the 35 HCC nodules (66%). In eight of these nodules, only very scarce ($<1\%$) tumoral cells displayed β-catenin positivity in the nucleus. In the other 15 cases, a strongly positive nuclear signal could be observed in 8 to 90% of tumoral nuclei (Figure 2B). β-catenin gene mutations were detected in nine of these 15 cases (Table 1). The mean rate of β-catenin-positive nuclei was significantly higher in HCCs carrying a mutated form of β-catenin than in HCCs without mutation (49.6 ± 11.4% *versus* 15.5 ± 6%, $P < 0.005$, Mann-Whitney test). Conversely, the number of HCCs with β-catenin mutation was significantly higher in the group of tumors harboring nuclear immunostaining for β-catenin in 8 to 90% of cells than in the group of tumors without β-catenin nuclear positivity (Table 2).

One tumor with a S45F mutation (Patient 4), and two HCC nodules carrying a β-catenin allele deleted of exon 3 sequences (Patients 11 and 12, Table 1) showed only

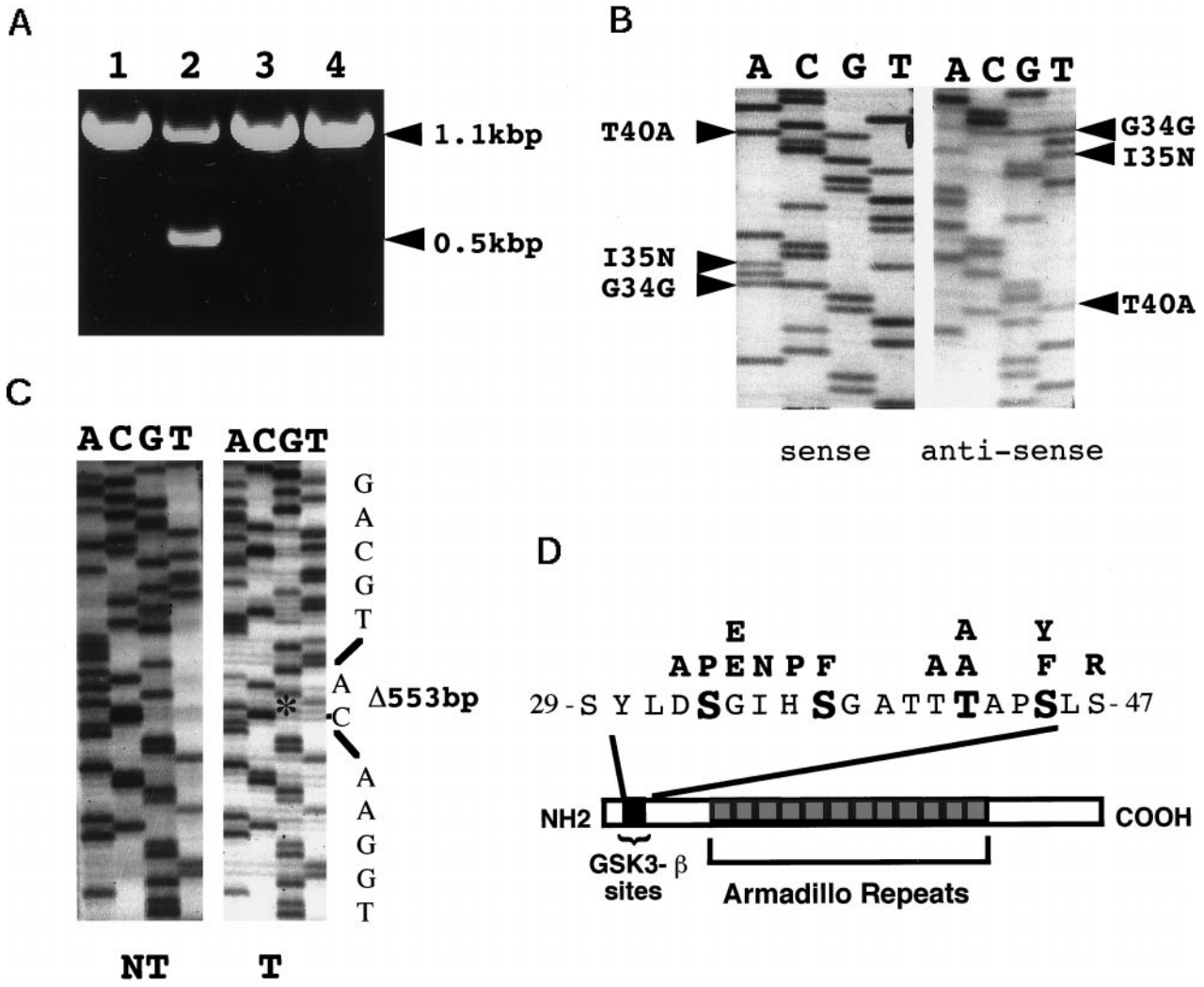

Figure 1. Oncogenic mutations of one β-catenin allele in HCC. A: Genomic PCR of β-catenin intron 2 to exon 4 sequences showing the normal PCR product (1.1 kbp) in tumors carrying wild-type (lanes 1 and 3) or point mutated β-catenin (lane 4, Patient 1) and a smaller product (0.5 kbp) corresponding to the deleted allele in Patient 12 (lane 2). B: Sequence analysis showing three point mutations in tumor from Patient 7. C: Sequence analysis of the tumorous (T) and nontumorous (NT) liver from Patient 11 showing an interstitial deletion spanning 553 bp of exon 3, intron 3, and exon 4. The dinucleotide CA was present at both ends of the deletion. D: Summary of amino acid substitutions found in 10 different tumors.

Table 1. Clinicopathological Data and Immunohistochemical Detection of Nuclear β-Catenin Expression in 12 Hepatocellular Carcinomas with β-Catenin Gene Alterations

Patient	Age/sex	Risk factor	Nontumoral liver	Tumor size (cm)	HCC grade	β-Catenin alterations	β-Catenin nu IS (%)
1	73/M	alcohol	cirrhosis	9	II	S45Y	60
2	66/M	alcohol	cirrhosis	3	II	H36P*	8
3	60/M	alcohol	fibrosis	17	II	S33P	90
4	71/M	alcohol	fibrosis	12	II	S45F	0
5	63/M	HCV	cirrhosis	5	I	G34E	65
6	61/F	HCV	cirrhosis	3	I	G34E	75
7	55/M	HBV	cirrhosis	19	I-II	T40A/I35N	90
8	63/M	hemochr	fibrosis	4	I	T41A	75
9	59/M	hemochr	normal	9	II	T41A/S47R	20
10	64/M	unknown	cirrhosis	14	II	D32A	90
11	50/M	unknown	normal	14	I	D20-144	<1
12	42/M	unknown	normal	6	I	D17-126	<1

*Only one of four tumoral nodules in the liver of Patient 2 carried a β-catenin mutation.

HCV, hepatitis C virus infection; HBV, hepatitis B virus infection; hemochr, genetic hemochromatosis; nu IS, nuclear immunostaining; %, percentage of β-catenin-positive nuclei (among 1000 tumor cells counted).

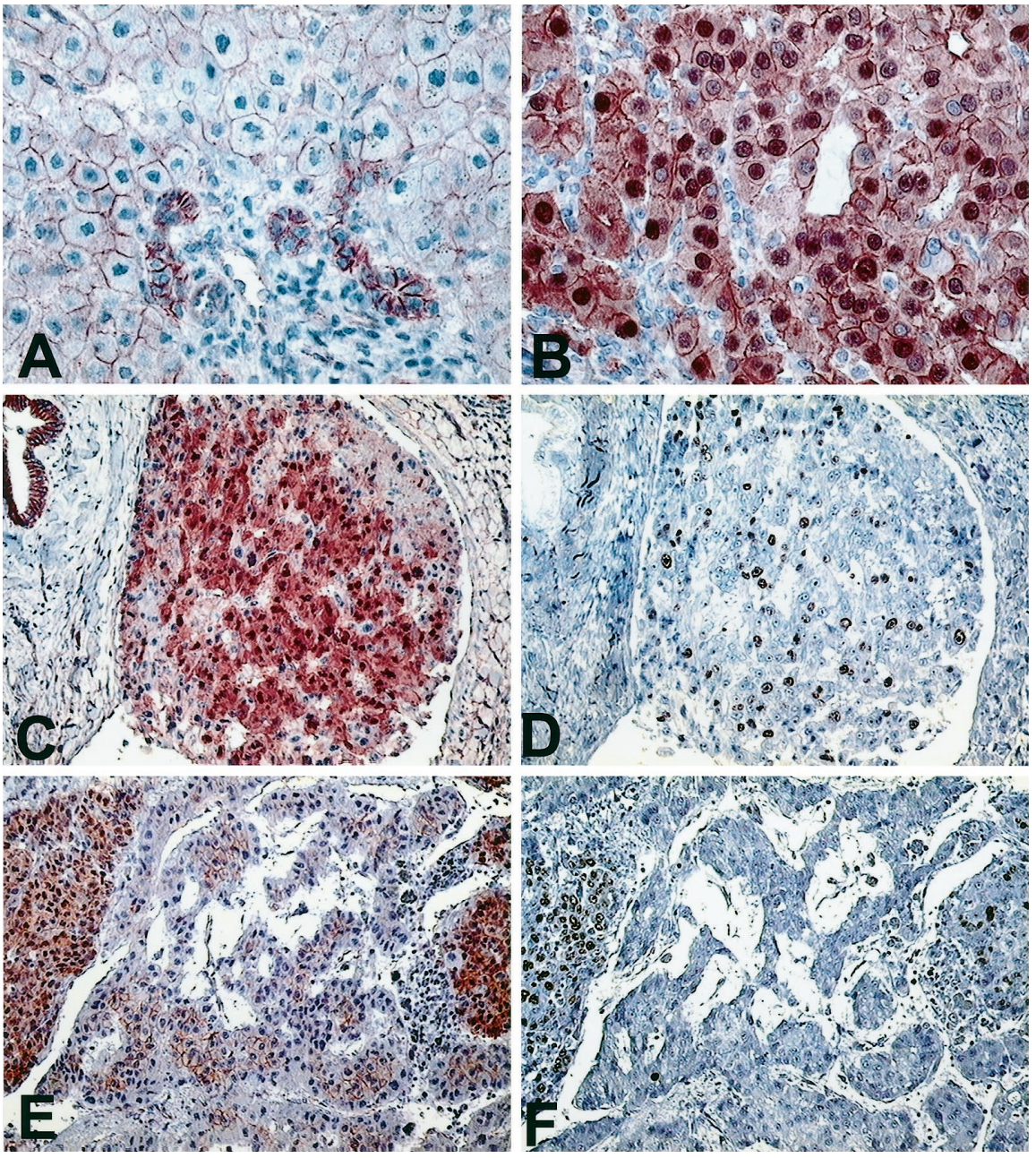

Figure 2. A, B, C, and E: β-catenin immunolocalization. A: In nontumoral liver, a thin membranous signal delineates the hepatocytes, and bile ductules show strong membranous and pale cytoplasmic staining. B: Overexpression of β-catenin in the cytoplasm and in most nuclei in a HCC case harboring a mutation in β-catenin exon 3. C: Tumoral invasion of a portal vein with strong cytoplasmic and nuclear β-catenin staining. E: Heterogeneous tumor showing strong cytoplasmic and nuclear β-catenin staining in most cells in some areas (left and right sides), and milder staining with only few positive nuclei in another area (center). D and F: MIB-1 (Ki-67) immunostaining. D: A serial section of the portal tumoral invasion shown in C, showing a high proliferative index. F: A serial section of the heterogeneous tumor in E, showing colocalization of MIB-1 and β-catenin stainings. Original magnifications, ×250 (A, B) and ×100 (C-F).

membranous β-catenin staining, with lower or equal intensity compared to corresponding nontumoral liver. On the other hand, four tumors in which β-catenin gene alteration could not be detected showed 30 to 80% of nuclei strongly positive for β-catenin staining. Further studies are required to determine whether these results reflect tumor heterogeneity, because different specimens were used for DNA analysis and for immunostaining. Finally, β-catenin expression was markedly elevated at the invasive front in three tumors showing otherwise moderate staining and in the tumoral intravascular compartment, compared with other tumor areas in 4 of the 9 cases of vascular invasion (Figure 2C).

Tumor Cell Proliferation

Proliferative index, determined by the count of tumoral nuclei that scored positive after immunostaining with a Ki-67 specific antibody, was twofold to more than 60-fold higher in tumors than in the nontumoral liver counterpart; the mean proliferative index was 498.5 (range, 12–1,854)

Table 2. Relationship between Nuclear Accumulation of β-Catenin and Mutations of the β-Catenin Gene or Proliferative Index in HCC Nodules

	Tumors with nuclear β-catenin staining* (n = 15)	Tumors with no nuclear β-catenin staining (n = 12)	P value
Tumors with β-catenin mutations	9 (60%)	1 (8%)	<0.005†
Proliferative index	603.4 ± 147.8	121.3 ± 46.6	<0.01‡

Mean values ± SE of the proliferative index are given as the counts of Ki-67-positive tumor cell nuclei in 10 consecutive high power (×400) fields.
*The percentage of nuclear β-catenin positive cells ranged from 8 to 90%.
†Fischer exact test.
‡Mann-Whitney U-test.

in tumors *versus* 18.1 (range, 0–100) in nontumoral liver. A higher number of positive cells was observed at the invasive front as well as in tumoral vascular invasion (Figure 2D). In some heterogeneous tumors, areas showing markedly elevated nuclear expression of β-catenin also exhibited increased number of Ki-67 positive cells (Figure 2, E and F). However, in four cases, the proliferative index was high (from 587 to 1,500 for 10 high power fields) in the absence of nuclear staining for β-catenin (data not shown).

Statistical analysis demonstrated a strong correlation between the rate at which cells expressed β-catenin in the nucleus and the number of Ki-67-positive cells ($P < 0.001$, Spearman test). When tumors were divided in two groups according to nuclear β-catenin staining, ie, from 8 to 90% of positive cells *versus* no nuclear positivity, a significant relationship between the two factors persisted ($P < 0.01$, Mann-Whitney test; Table 2). In contrast, no significant relationship could be established between the β-catenin gene status and the proliferative index.

Correlation with Clinical Course and Disease Status

Clinicopathological parameters were collected for all 32 patients with a median follow-up of 20 months (range, 8–72 months). Recurrence of HCC occurred in 11 patients (within one year after surgery in 9 of them) and was responsible for death in 6 of these 9 cases. In two cases, recurrence was detected at the 24th month. Two patients for whom follow-up was shorter than 12 months and seven patients who died in the immediate postoperative period were excluded from statistical analysis. Although neither survival nor recurrence rates could be definitely established because of the short mean follow-up time, statistical analysis suggested a strong correlation between high cell proliferation index in the primary tumor and poor survival. Indeed, patients who died by recurrence (n = 6) had a much higher proliferative index in resected primary HCC than patients still alive (n = 15; mean value 1,135.67 ± 304.32 *versus* 288.07 ± 77.78, $P < 0.02$). High proliferation index in the primary tumor also correlated with tumor recurrence (Table 3). In addition, recurrence was related to a high rate of cells expressing β-catenin in their nuclei; tumor recurrence was observed in 10 cases in which the mean rate of positive nuclei for β-catenin was high, whereas 11 patients harboring primary HCCs with lower rate of β-catenin-positive nuclei remained free of disease (Table 3). These results need to be confirmed by a longer follow-up.

Finally, recurrence tended to correlate with large tumor size ($P = 0.06$) but not with HCC histological grading or vascular invasion.

Discussion

Deregulated expression of β-catenin, which may result from APC defects, activating mutations in the β-catenin gene itself, or other alterations in the Wnt pathway, has been implicated as an important step in carcinogenesis. In the present study, an immunohistochemical approach was used to assess the prevalence of Wnt/β-catenin pathway activation in human HCC and to evaluate the impact of somatic mutations affecting the regulatory region of β-catenin on the expression and cellular localization of the protein. β-catenin immunostaining showed increased membranous and cytoplasmic expression in most HCCs by comparison with nontumoral hepatocytes, as already described in a previous study.[27] More importantly, abnormal nuclear localization of β-catenin could be demonstrated in 15 of 35 tumors analyzed (43%). However, in all these tumors, only a fraction of the cells was labeled (from 8 to 90%), and we found additional cases in which only few isolated tumor cells (<1%) harbored nuclear β-catenin staining, thus suggesting several hypotheses. First, delocalization of β-catenin to the nucleus might depend on specific intracellular conditions that may vary between cells in the same tumoral area. Second, neighboring cells could be genetically hetero-

Table 3. Relationship between Tumor Recurrence and Nuclear Accumulation of β-Catenin or Proliferative Index in Primary HCCs

	Patients with recurrence (n = 10)	Patients free of disease (n = 11)	P value*
Nuclear β-catenin rate	44.27 ± 12.28%	18.2 ± 9.91%	<0.05
Proliferative index	880.6 ± 217.15	211.73 ± 70.11	<0.02

Nuclear immunostaining rates are given as the mean ± SE percentage of β-catenin-positive nuclei. Proliferative indexes are given as the mean ± SE count of Ki-67-positive cells in 10 consecutive high power (×400) fields.
*Mann-Whitney U-test.

geneous and β-catenin activation might occur during HCC progression, as shown in human prostate cancer.[28] This notion is supported by the finding of very scarce labeled nuclei in a significant proportion of tumors, and by the complete absence of nuclear staining in cirrhotic nodules and in dysplastic lesions associated with chronic hepatitis, which might represent preneoplastic conditions for HCC development. In addition, it has been recently shown that differences in specimen preparation and/or immunostaining protocols may strongly affect the detection of nuclear β-catenin in melanoma cells.[23] In this study, we used an improved antigen retrieval method (see Materials and Methods) allowing more efficient detection of the nuclear signals, which might explain the discrepancy between our data and those reported in a previous analysis of 44 HCC samples.[27]

Our results, showing a strong correlation between nuclear β-catenin staining and somatic mutations of the β-catenin gene in HCC, indicate that activation of the Wnt/β-catenin pathway in HCC occurs predominantly through activating mutations in the β-catenin gene itself. It differs from colorectal cancers, in which APC mutations are responsible for β-catenin stabilization in 80% of the cases,[29] and from melanoma or ovarian and uterine carcinomas, in which very occasional mutations can be found despite frequent β-catenin overexpression.[21–23] In our study, 34% of tumor samples had mutations or deletions in the third exon of the β-catenin gene. Similar mutation rates were reported in previous studies of European and Asian patients with HCC, albeit with some differences in the distribution of mutated amino acids.[8,9] Further studies of the functional role of these different mutations on β-catenin stability might be important to better define the mechanisms involved in the posttranscriptional control of β-catenin expression. In addition, intense nuclear β-catenin expression in HCCs carrying apparently normal β-catenin alleles (11% of the cases) might be associated with alterations of the β-catenin gene in a region other than exon 3 or with defects in the APC gene, although the very low prevalence of allelic losses at the APC locus on chromosome 5q21 in HCC does not favor the latter hypothesis.[3,30] Another possible mechanism is the involvement of other factors mediating Wnt signaling, like the human frizzled (Fz) genes. Indeed, a novel Fz gene (FzE3) has recently been shown to enhance β-catenin signals in human esophageal carcinomas.[31]

Nuclear translocation of β-catenin and its association with Tcf/Lef-1 factors can activate gene expression and cell proliferation in experimental systems.[14] The present study revealed a significant relationship between the number of tumoral hepatocytes with nuclear β-catenin and the number of Ki-67-positive cells. To the best of our knowledge, this is the first *in vivo* study demonstrating a link between the stabilization and nuclear accumulation of β-catenin and the proliferation of tumor cells. Whether c-*myc* or other target genes of Wnt/β-catenin signaling play a direct role in this process is an important issue. However, our data showing a very high proliferative index in four HCC cases in the absence of β-catenin mutation and nuclear expression, provide evidence implicating other mechanisms in active tumor cell proliferation. It is of interest that high cell proliferation in the primary tumor has been closely related to poor survival and tumor recurrence in HCC patients in previous reports,[32,33] as also suggested in the present study. In addition, our preliminary data seem to indicate that nuclear accumulation of mutated β-catenin in HCC might be associated with an increased risk of tumor recurrence and poor survival. However, the mean follow-up was short in the present work; larger studies with longer follow-up are clearly needed to confirm these data and to determine whether oncogenic activation of β-catenin and active cell proliferation represent independent prognostic factors in HCC.

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References

1. Murray CJL, Lopez AD: Mortality by cause for eight regions of the world: global burden of disease study. Lancet 1997, 349:1269–1276
2. Nagai H, Buendia MA: Oncogenes, tumor suppressors, and cofactors in hepatocellular carcinoma. Hepatitis B Virus. Edited by Koshy R, Caselman W. London, Imperial College Press, 1998, pp 182–218
3. Nagai H, Pineau P, Tiollais P, Buendia MA, Dejean A: Comprehensive allelotyping of human hepatocellular carcinoma. Oncogene 1997, 14:2927–2933
4. Murakami Y, Hayashi K, Hirohashi S, Sekiya T: Aberrations of the tumor suppressor p53 and retinoblastoma genes in human hepatocellular carcinomas. Cancer Res 1991, 51:5520–5525
5. Hsu HC, Peng SY, Lai PL, Sheu JC, Chen DS, Lin LI, Slagle BL, Butel JS: Allelotype and loss of heterozygosity of p53 in primary and recurrent hepatocellular carcinomas. Cancer 1994, 73:42–47
6. De Souza AT, Hankins GR, Washington MK, Orton TC, Jirtle RL: M6P/IGF2R gene is mutated in human hepatocellular carcinomas with loss of heterozygosity. Nat Genet 1995, 11:447–449
7. Nishida N, Fukuda Y, Komeda T, Kita R, Sando T, Furukawa M, Amenomori M, Shibagaki I, Nakao K, Ikenaga M, Ishizaki K: Amplification and overexpression of the cyclin D1 gene in aggressive hepatocellular carcinoma. Cancer Res 1994, 54:3107–3110
8. de La Coste A, Romagnolo B, Billuart P, Renard CA, Buendia MA, Soubrane O, Fabre M, Chelly J, Beldjord C, Kahn A, Perret C: Somatic mutations of the β-catenin gene are frequent in mouse and human hepatocellular carcinomas. Proc Natl Acad Sci USA 1998, 95:8847–8851
9. Miyoshi Y, Iwao K, Nagasawa Y, Aihara T, Sasaki Y, Imaoka S, Murata M, Shimano T, Nakamura Y: Activation of the β-catenin gene in primary hepatocellular carcinoma by somatic alterations involving exon 3. Cancer Res 1998, 58:2524–2527
10. He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B, Kinzler KW: Identification of c-myc as a target of the APC pathway. Science 1998, 281:1509–1512
11. Tetsu O, McCormick F: β-Catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature 1999, 398:422–426
12. Aberle H, Schwartz H, Kemler R: Cadherin-catenin complex: protein interactions and their implications for cadherin function. J Cell Biochem 1996, 61:514–523
13. Cadigan KM, Nusse R: Wnt signaling: a common theme in animal development. Genes Dev 1997, 11:3286–3305
14. Polakis P: The adenomatous polyposis coli (APC) tumor suppressor. Biochim Biophys Acta 1997, 1332:F127–147
15. Clevers H, van de Wetering M: TCF/LEF factors earn their wings. Trends Genet 1997, 13:485–489

16. Pennica D, Swanson TA, Welsh JW, Roy MA, Lawrence DA, Lee J, Brush J, Taneyhill LA, Deuel B, Lew M, Watanabe C, Cohen RL, Melhem MF, Finley GG, Quirke P, Goddard AD, Hillan KJ, Gurney AL, Botstein D, Levine AJ: WISP genes are members of the connective tissue growth factor family that are up-regulated in wnt-1-transformed cells and aberrantly expressed in human colon tumors. Proc Natl Acad Sci USA 1998, 95:14717–14722
17. Morin PJ, Sparks AB, Korinek V, Barker N, Clevers H, Vogelstein B, Kinzler KW: Activation of β-catenin-Tcf signaling in colon cancer by mutations in β-catenin or APC. Science 1997, 275:1787–1790
18. Iwao K, Nakamori S, Kameyama M, Imaoka S, Kinoshita M, Fukui T, Ishiguro S, Nakamura Y, Miyoshi Y: Activation of the β-catenin gene by interstitial deletions involving exon 3 in primary colorectal carcinomas without adenomatous polyposis coli mutations. Cancer Res 1998, 58:1021–1026
19. Polakis P: The oncogenic activation of β-catenin. Curr Opin Genet Dev 1999, 9:15–21
20. Inomata M, Ochiai A, Akimoto S, Kitano S, Hirohashi S: Alteration of β-catenin expression in colonic epithelial cells of familial adenomatous polyposis patients. Cancer Res 1996, 56:2213–2217
21. Palacios J, Gamallo C: Mutations in the β-catenin gene (CTNNB1) in endometrioid ovarian carcinomas. Cancer Res 1998, 58:1344–1347
22. Fukuchi T, Sakamoto M, Tsuda H, Maruyama K, Nozawa S, Hirohashi S: Beta-catenin mutation in carcinoma of the uterine endometrium. Cancer Res 1998, 58:3526–3528
23. Rimm DL, Caca K, Hu G, Harrison FB, Fearon ER: Frequent nuclear/cytoplasmic localization of β-catenin without exon 3 mutations in malignant melanoma. Am J Pathol 1999, 154:325–329
24. Pileri S, Roncador G, Ceccarelli C, Piccioli M, Briskomatis A, Sabattini E, Ascani S, Santini D, Piccaluga P, Leone O, Damiani S, Ercolessi C, Sandri F, Pieri F, Leoncini L, Falini B: Antigen retrieval techniques in immunohistochemistry: comparison of different methods. J Pathol 1997, 183:116–123
25. Barnes D, Gillett C: Determination of cell proliferation. J Clin Pathol Clin Mol Pathol 1995, 48:M2–M5
26. Ishak K, Anthony P, Sobin L: Histological Typing of Tumors of the Liver. Berlin, Springer Verlag, 1994
27. Ihara A, Koizumi H, Hashizume R, Uchikoshi T: Expression of epithelial cadherin and α- and β-catenins in nontumoral livers and hepatocellular carcinoma. Hepatology 1996, 23:1441–1447
28. Voeller HJ, Truica CI, Gelmann EP: Beta-catenin mutations in human prostate cancer. Cancer Res 1998, 58:2520–2523
29. Rubinfeld B, Albert I, Porfiri E, Fiol C, Munemitsu S, Polakis P: Binding of GSK3β to the APC-β-catenin complex and regulation of complex assembly. Science 1996, 272:1023–1026
30. Ding SF, Delhanty JD, Dooley JS, Bowles L, Wood CB, Habib NA: The putative tumor suppressor gene on chromosome 5q for hepatocellular carcinoma is distinct from the MCC and APC genes. Cancer Detect Prev 1993, 17:405–409
31. Tanaka S, Akiyoshi T, Mori M, Wands J, Sugimachi K: A novel frizzled gene identified in human esophageal carcinoma mediates APC/β-catenin signals. Proc Natl Acad Sci USA 1998, 95:10164–10169
32. Kitamoto M, Nakanishi T, Kira S, Kawaguchi M, Nakashio R, Suemori S, Kajiyama G, Asahara T, Dohi K: The assessment of proliferating cell nuclear antigen immunohistochemical staining in small hepatocellular carcinoma and its relationship to histologic characteristics and prognosis. Cancer 1993, 72:1859–1865
33. Tannapfel A, Geissler F, Köckerling F, Katalinic A, Hauss J, Wittekind C: Apoptosis and proliferation in relation to histopathological variables and prognosis in hepatocellular carcinoma. J Pathol 1999, 187:439–445