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Childhood Hepatoblastomas Frequently Carry a Mutated Degradation Targeting Box of the β-Catenin Gene[1]

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Abstract

Hepatoblastomas (HBs) are embryonal tumors affecting young children and representing the most frequent malignant liver tumors in childhood. The molecular pathogenesis of HB is poorly understood. Although most cases are sporadic, the incidence is highly elevated in patients with familial adenomatous polyposis coli. These patients carry germline mutations of the *APC* tumor suppressor gene. APC controls the degradation of the oncogene product β-catenin after its NH_2-terminal phosphorylation on serine/threonine residues. APC, as well as β-catenin, has been found to be a central effector of the growth promoting *wingless* signaling pathway in development. To find out if this pathway is involved in the pathogenesis of sporadic HBs, we examined 52 biopsies and three cell lines from sporadic HBs for mutations in the *APC* and *β-catenin* genes. Using single-strand conformational polymorphism analysis, deletion screening by PCR, and direct sequencing, we found a high frequency of β-catenin mutations in sporadic HBs (48%). The mutations affected exon 3 encoding the degradation targeting box of β-catenin leading to accumulation of intracytoplasmic and nuclear β-catenin protein. The high frequency of activating mutations in the *β-catenin* gene indicates an important role in the pathogenesis of HB.

Introduction

HBs[3] are malignant embryonal liver tumors mainly affecting young children in the first 3 years of life (1). Histologically, HBs are composed of immature epithelial cells resembling immature, developing liver during development and sometimes also contain immature mesenchymal or teratomatous elements (1). The molecular pathogenesis is poorly understood. Regions with allelic loss have been described in HB on chromosomes 1 and 11, however, candidate genes have not been identified (2, 3). The incidence of this tumor is elevated in patients with FAP, which is known to carry germline mutations in the *APC* gene (4–6).

The *APC* gene product is part of a multiprotein complex and regulates the cytoplasmic level of β-catenin by direct binding to β-catenin (7) and promotes its NH_2-terminal phosphorylation by GSK-3β, thereby targeting β-catenin for degradation by the proteasome system (8). β-catenin is important for the process of cell-cell adhesion (9, 10) and is also the central effector molecule of the *wingless/WNT* developmental signaling pathway (7). It can enter the cell nucleus together with Tcf-lymphoid enhancer factors, and regulates transcription of target genes, including *c-myc* of this signaling pathway (7, 11, 12). In colon carcinomas, both inactivating *APC* mutations and activating mutations of *β-catenin* result in an increased cytoplasmic β-catenin level with oncogenic activity. In a murine transgenic model carrying a truncated *β-catenin* gene, an increased cellular turnover of intestinal epithelial cells has been demonstrated (13). Because of the increased incidence of HB in FAP patients we postulated that the APC/β-catenin pathway may also be altered in sporadic HBs.

Materials and Methods

Patients, Tumors, and Cell Lines. A total of 52 HB biopsies and three HB cell lines from sporadic HBs were analyzed. Two of the HB cell lines have been published previously (HUH6, HepT1; Refs. 14, 15). In addition, we tested one biopsy sample and the cell line HepG2, both derived from childhood HCC. HepG2 (16) was obtained from American Type Culture Collection (Manassas, VA), and HuH6 was obtained from the Japanese Collection of Research Biosources (Osaka, Japan). The HepT3 cell line was derived from tumor D204, and the HepT1 cell line was derived from tumor DZ25. In two cases (HepT1 and HepT3), we were able to study both the primary tumor and the cell line. From 32 HB samples, sufficient tumor material was available for RNA isolation. Constitutional genomic DNA was isolated in all cases either from peripheral blood leukocytes or liver tissue adjacent to the tumor. The age of patients ranged from 4–57 months.

DNA and cDNA Preparation. DNA was extracted from HB samples, peripheral blood, and normal liver tissue by standard proteinase K digestion and phenol/chloroform extraction (17). Total cellular RNA was prepared by lysis in guanidinium isocyanate and ultracentrifugation through a cesium chloride cushion or with the Trizol reagent (Life Technologies, Inc.), according to the protocols of the supplier. Contaminating residual genomic DNA was removed by digestion with RNase-free DNase (Boehringer Mannheim) before reverse transcription. The RNAs were reverse transcribed using the SuperScript Preamplification System (Life Technologies, Inc.) with random hexamers as primers. Before DNA or RNA extraction, individual tissue samples were preexamined by frozen section histology to document the histopathological appearance of the specimen.

Mutational Analyses of the *β-Catenin* Gene. Genomic DNA from each tumor sample was amplified for SSCP analysis of exon 3 using the following primer pair (BCAT-1/BCAT-2): exon 3, 5′-GATTTGATGGAGTTGGACATGG-3′ and 5′-TGTTCTTGAGTGAAGGACTGAG-3′. Samples were amplified through 35 cycles on a Uno Thermoblock cycler (Biometra) at 94°C denaturation for 35 s, 63°C annealing for 35 s, and 72°C extension for 40 s. PCR reactions were performed in a volume of 10 μl with 20 ng of genomic DNA in a PCR buffer containing 1.5 mM $MgCl_2$ (Life Technologies, Inc.), 200 μM each deoxyribonucleoside triphosphate, 5 pmol of each primer, and 0.25 unit of Taq polymerase (Life Technologies, Inc.). After PCR amplification, products were loaded onto 10% and 14% polyacrylamide gels with different acrylamide:bisacryl-amide ratios (1:99, 1:29), with and without 5% glycerol at RT. The single and double strands of the PCR products were visualized by silver staining, as described previously (18). PCR products that showed a gel mobility shift were excised from the wet gel, eluted, and reamplified. The resulting PCR products were purified using spin columns (QIAquick PCR purification kit; Qiagen, Inc.), and sequenced. Genomic tumor DNA, which

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[3] The abbreviations used are: HB, hepatoblastoma; APC, adenomatous polyposis coli; FAP, familial adenomatous polyposis; GSK-3β, glycogen synthase kinase-3β; HCC, hepatocellular carcinoma; RT, room temperature; RT-PCR, reverse transcription-PCR; SSCP, single-strand conformational polymorphism.

showed no aberrant bands on SSCP, were directly sequenced. PCR was performed with the same primers and protocol as described for the SSCP analysis. PCR products were purified using spin columns (QIAquick PCR purification kit; Qiagen, Inc.) and sequenced, as described below.

For the detection of deletions, cDNA and genomic DNA from tumor and normal liver tissue was amplified using the primer pair BCAT-3/BCAT-4, flanking exon 3, and the primer pair BCAT-5/BCAT-6, flanking exons 2–4, of the *β-catenin* gene: BCAT-3, 5′-AAAATCCAGCGTGGACAATGG-3′; BCAT-4, 5′-TGTGGCAAGTTCTGCATCATC-3′: BCAT-5, 5′-GGAGGA-AGGTCTGAGGAGCAG-3′; BCAT-6, 5′-CGATGATGGGAAAGGTTAT-GC-3′. The PCR was carried out on an UnoII Thermoblock cycler (Biometra) with an initial denaturation step at 94°C for 3 min; 42 cycles at 94°C for 40 s, 54°c for 40 s, and 72°C for 50 s; and a final extension cycle at 72°C for 10 min. Each reaction contained 1 μl of cDNA template, 5 pmol forward and reverse primers, 200 μM each deoxyribonucleoside triphosphate, and a PCR buffer (Life Technologies, Inc.) containing 1.0 mM $MgCl_2$ and 1% DMSO. PCR products were visualized on a 2% agarose gel with ethidium bromide. PCR products of altered size were extracted and purified from agarose gel with QIAquick Gel Extraction kit (Qiagen, Inc.).

Cycle sequencing using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) was done on a TC 9600 Thermocycler (Perkin-Elmer Corp.), using 20 ng of PCR or RT-PCR product as template. Each sequencing product was run on an ABI 373A sequencer (Applied Biosystems).

Western Blot Analysis. HB samples were analyzed for β-catenin protein expression by Western blotting. Frozen sections of the tumor samples were examined microscopically to exclude the presence of necrotic tissue or contaminating nonneoplastic liver tissue. One hundred fifty 5-μm cryostat tumor sections were cut at −25°C, collected in liquid nitrogen, and lysed for 30 min on ice in 500 μl of ice-cold lysis buffer consisting of 20 mM Tris-HCl (pH 7.4), 50 mM NaCl, 1% NP40, with 1 mM phenylmethylsulfonylfluoride, 10 μg/ml leupeptin (Boehringer Mannheim), and 100 units/ml aprotinin (Calbiochem). Debris was removed by centrifugation for 10 min. at 13,000 × *g* at 4°C. Protein concentrations were determined using a DC protein assay (Bio-Rad). Soluble protein (10 μg) was separated by electrophoresis on 15% SDS-polyacrylamide gels and blotted onto nitrocellulose. After blocking with 5% nonfat dry milk in PBS for 2 h at RT, the filters were incubated with anti-β-catenin antibodies (clone 14, IgG1, 0.5 μg/ml in PBS, 0.1% BSA, Transduction; and clone 7D11, IgG2a, 1 μg/ml in PBS, 0.1% BSA, Alexis). The latter detects an epitope located in the NH_2-terminal degradation targeting box of-β-catenin. Binding of the primary antibody was detected by alkaline phosphatase antialkaline phosphatase staining (19). The filters were developed using nitroblue tetrazolium/bromo-chloro-indolyl phosphate substrate.

Immunohistochemistry. Sections from formalin-fixed paraffin-embedded tumor samples were cut at 4 μm, mounted on positively charged slides (Superfrost + Menzel), air-dried in an incubator at 42°C overnight, and deparaffinized in xylene. After rehydration in graded alcohols, the slides were incubated in 1% hydrogen peroxide diluted in methanol for 30 min to block endogenous peroxidase activity, and then rehydrated in distilled water, followed by PBS. After microwave treatment for 30 min in 0.1 M sodium citrate (pH 6.0), the slides were incubated in a blocking solution (PBS with 5% nonfat dry milk and 2% normal rabbit serum) for 30 min at RT. This was followed by a 2 × 15-min incubation with avidin-biotin blocking solutions (avidin-biotin blocking kit; Vector Laboratories, Inc.). The solution was removed from the slides using a filter paper, and the monoclonal anti-β-catenin antibody 14 (IgG1, 0.25 μg/ml in PBS, 0.1% BSA; Transduction) was added to the samples overnight at 4°C. After removing unbound antibody by several rinses with PBS and PBS containing 0.1% Triton X100, the bound antibody was detected using the avidin-biotin complex method (DAKO) and visualized by diaminobenzidine tetrahydrochloride. Slides were lightly counterstained with hematoxylin.

Results and Discussion

Inactivating mutations of the second *APC* allele have been found in HBs of FAP patients (20). Thus, we screened sporadic HBs for *APC* mutations, but were not able to detect mutations in the mutation cluster region of *APC* (6) in this collective of HBs (data not shown). Nontruncating point mutations leading to amino acid substitutions of APC have been described in HB of Asian patients (21). The apparent lack of *APC* mutations in our series may be related to the different genetic background of our patients. We then screened 52 primary HB tumor samples and three HB cell lines for mutations in the *β-catenin* gene as an important complex partner of APC. Using SSCP analysis, we analyzed a 228-bp fragment of exon 3 of the *β-catenin* gene, in

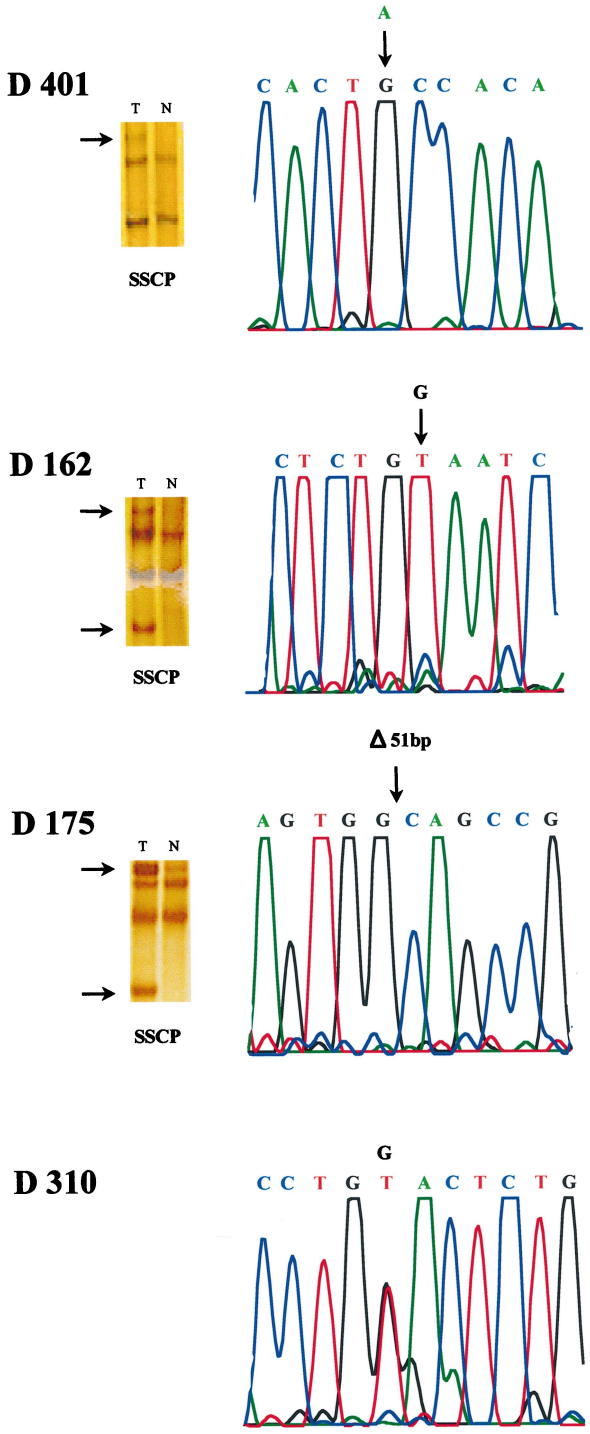

Fig. 1. *β-catenin* mutations in HBs D162, D401, D175, and D310. The first three mutations were identified by SSCP screening. Aberrant bands are indicated by *arrows*. DNA sequencing of the excised and reamplified DNA products of tumor D401 uncovers a somatic A→G transition in codon 41, representing the most frequent point mutation of β-catenin in HB leading to a substitution of threonine by alanine. Tumor D162 carries a G→T transversion in tumor D162, resulting in a glycine to valine amino acid change in codon 34. In tumor D 175, a 51-bp in-frame deletion in exon 3 was found. This alteration causes a 17 amino acid deletion (codons 22–38). D310 had no bandshift in SSCP analysis. A mutation of codon 32 (Asp32Tyr) was detected by direct sequencing.

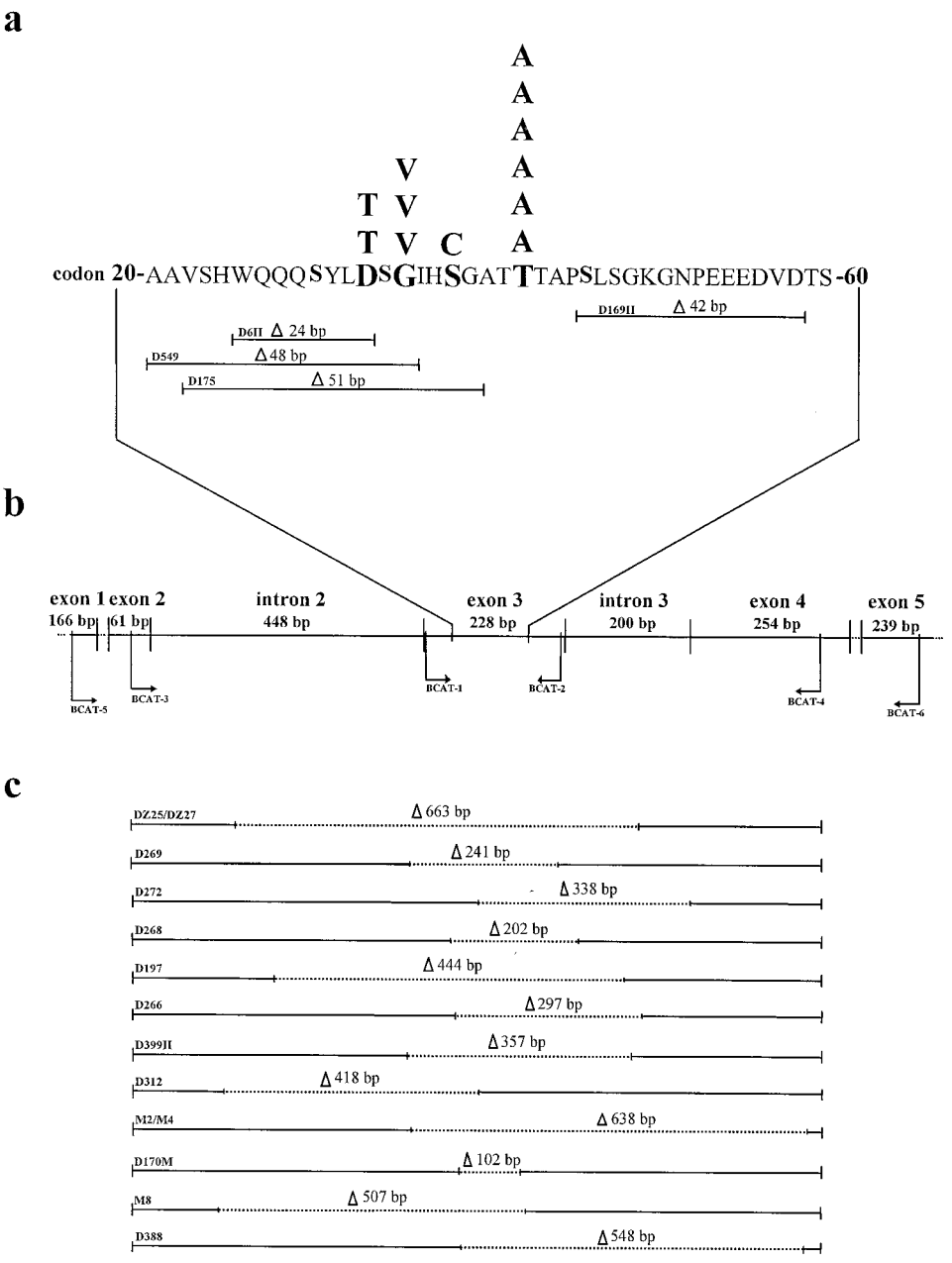

Fig. 2. Schematic illustration of the location of *β-catenin* mutations in HB. *a*, point mutations and small deletions identified in the regulatory degradation targeting box encoded by exon 3. *Bold type* indicates mutated amino acids, as well as potential phosphorylation sites on threonine and serine residues. Amino acid substitutions indicated above. *b*, schematic diagram of exons 1–5 of the *β-catenin* gene with the location of the primers used for SSCP screening, sequencing, and detection of deletions. *c*, location of the larger deletions spanning exon 3 or disrupting exon-intron boundaries.

which several serine and threonine residues are located that serve as targets for phosphorylation by GSK-3β. We found PCR products with altered mobility in 14 of 52 HBs and in two HB cell lines. Sequencing of these bands uncovered 11 point mutations and five small interstitial deletions of 24–102 bp (Table 1; Figs. 1 and 2). Two further cases with a point mutation (Asp32Tyr) were identified by direct sequencing of exon 3 in tumors without SSCP shift. Eight point mutations abolished serine or threonine phosphorylation sites. An increased transcriptional activity has been demonstrated *in vitro* for β-catenin forms carrying similar mutations in exon 3 (11).

Five mutations resulted in amino acid substitutions in codons 32 and 34, suggesting an influence on the accessibility of the serine 33 for the GSK-3β kinase, preventing its phosphorylation (Fig. 2*a*). The predominant mutation was an A→G transition in codon 41 in six tumors, resulting in a substitution of threonine by alanine. Codon 41 mutations have also been described in colorectal carcinomas at lower frequency (22). In contrast, the most frequent point mutation in colorectal carcinoma, Ser45Phe, was absent in HBs. The four deletions clustered in the NH_2-terminal regulatory domain of the *β-catenin* gene (codons 29–45). They affected the NH_2-terminal phosphorylation sites necessary for targeting the protein for degradation (Fig. 2*a*). In melanoma cell lines and in colorectal cancers, larger deletions have been described previously (23). Therefore, we screened the HB panel for larger deletions with primers spanning exon 3 and the adjacent introns. Genomic DNA from HBs was amplified, and PCR products with reduced size were identified in nine HB biopsies and one cell line (Table 1; Fig. 3). The bands were excised, purified, and sequenced. Large deletions varying from 202–663 bp were identified, most of them deleting the whole exon 3, and in one case sequences from exon 4, also (Fig. 2*c*). The deletion of sequences or disruption of the splice sites of exon 3 resulted in altered transcripts and in β-catenin proteins without a functional NH_2-terminal degradation box. We were able to confirm these large deletions by RT-PCR in 8 of 32 HBs from which cDNA was available (Fig. 3). Sequencing showed tran-

Table 1 *Summary of clinical data and mutations of β-catenin in HBs*

Case	Age (mo)/sex	Chemotherapy	Histology	Nucleotide change	Codon	Effect of mutation
D422	19/M	No	Epithelial	GAC to TAC	32	Asp 32 Tyr
D310	30/M	No	Mixed	GAC to TAC	32	Asp 32 Tyr
D162	19/M	Yes	Epithelial, multifocal	GGA to GTA	34	Gly 34 Val
D306	12/M	No	Epithelial	GGA to GTA	34	Gly 34 Val
D316	10/M	No	Epithelial	GGA to GTA	34	Gly 34 Val
M16	36/M	No	Epithelial	TCT to TGT	37	Ser 37 Cys
D104	27/F	No	Epithelial	ACC to GCC	41	Thr 41 Ala
D204	9/M	No	Mixed	ACC to GCC	41	Thr 41 Ala
D565	18/F	Yes	Mixed	ACC to GCC	41	Thr 41 Ala
D401	9/F	Yes	Mixed	ACC to GCC	41	Thr 41 Ala
D379	4/F	Yes	Mixed	ACC to GCC	41	Thr 41 Ala
D339 (cell line HUH6)	12/M	No	Mixed	ACC to GCC	41	Thr 41 Ala
D501 (cell line of D204)	9/M	No	Epithelial	ACC to GCC	41	Thr 41 Ala
D6II	11/F	No	Epithelial	Δ24 bp	25–32	Δ8 aa in exon 3
D169II	40/F	No	Small cell, anaplastic	Δ42 bp	45–58	Δ14 aa in exon 3
D549	29/M	Yes	Mixed	Δ48 bp	20–35	Δ16 aa in exon 3, Lys 19 Asp
D175	16/M	No	Epithelial	Δ51 bp	22–38	Δ17 aa in exon 3
D170M	11/M	Yes	Epithelial	Δ102 bp	28–61	Δ34 aa in exon 3
DZ25	34/F	No	Epithelial, multifocal	Δ663 bp	5–80	Δ76 aa, exon 3
DZ27 (cell line of DZ25)	34/F	No	Epithelial	Δ663 bp	5–80	Δ76 aa, exon 3
D269	9/F	No	Mixed	Δ241 bp	5–80	Δ76 aa, exon 3
D272	12/M	No	Mixed	Δ338 bp	35–80	Δ46 aa, exon 3
D268/D158	7/F	No	Mixed	Δ202 bp	5–80	Δ76 aa, exon 3
D197	54/M	Yes	Epithelial	Δ444 bp	5–80	Δ76 aa, exon 3
D266	8/F	No	Mixed	Δ297 bp	5–80	Δ76 aa, exon 3
D399II	10/M	No	Epithelial	Δ357 bp	5–80	Δ76 aa, exon 3
D312	15/F	No	Mixed	Δ451 bp, ins 33 bp	5–35	Δ31 aa, exon 3
M2	3 yr/M	No	Mixed: epithelial	Δ638 bp	5–141	Δ137 aa, Ala 5 Asp, exon 3, exon 4
M4	3 yr/M	No	Mixed: teratomatous	Δ638 bp	5–141	Δ137 aa, Ala 5 Asp, exon 3, exon 4
M8	6 yr/M	No	HCC	Δ507 bp	5–59	Δ55 aa, exon 3
D388 (cell line HepG2)	15 yr/M	No	HCC	Δ548 bp	25–140	Δ116 aa, exon 3, exon 4

scripts with in-frame deletions resulting in smaller proteins with NH_2-terminal deletions. In the majority of HB cases, constitutional DNA from normal liver tissue or peripheral blood lymphocytes was available. All *β-catenin* mutations were somatic, and the constitutional DNA showed no alterations (for example see Fig. 3). In one case (M2/M4), microdissected areas with different histological features (epithelial and teratomatous, respectively) were shown to carry identical *β-catenin* mutations, providing evidence for a clonal origin of both components. Overall, sporadic HBs showed a mutation frequency of 48%. Therefore, HB is the malignancy with the highest mutation frequency of all tumor types known to carry oncogenic *β-catenin* mutations. In other primary tumors such as colorectal carcinomas, *β-catenin* mutations occur in <25% (11, 22, 24–28). In HB, there was no correlation with a particular histological type, preoperative chemotherapy, or clinical outcome (Table 1, and data not shown). Interestingly, all three HB cell lines tested carried *β-catenin* mutations. Two of the cell lines were established in our laboratory so that the primary tumor material was also available, showing identical mutations. Thus, *β-catenin* mutations are most likely not cell culture-induced, but the presence of such a genetic lesion may facilitate the primary outgrowth and long-term passage in culture.

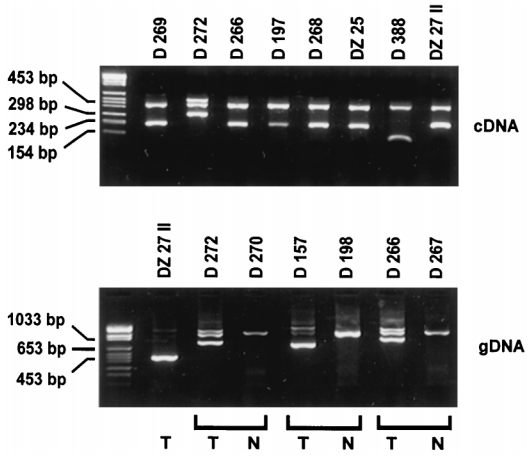

Fig. 3. Deletions in the *β-catenin* gene in HBs detected by RT-PCR and PCR of genomic DNA with primers spanning exon 3 (BCAT3/4). In addition to the wild type product of 467 bp with cDNA (*top*) and 1115 bp with genomic DNA (*bottom*), smaller products representing the deleted *β-catenin* gene product can be detected. In three cases, the corresponding constitutive gDNA (*Lane N*) yields a PCR product of the expected size and, therefore, shows no evidence of deletion, whereas tumor gDNA (*Lane T*) yields shortened products indicating deletions. Interestingly, amplification of tumor gDNA also yielded products that were larger than the wild type *β-catenin* product suggesting that the deletions originate from complex duplication/deletion mutations. However, the larger variants did not result in larger transcripts, except for D272. The exact nature of these bands is presently under study.

Mutations in the degradation targeting box should lead to increased levels of the β-catenin protein. Western blot analysis demonstrated cellular accumulation of mutated β-catenin proteins in HB samples (Fig. 4, *a* and *b*). Accumulation was also seen by immunohistochemistry (Fig. 4*c*). Tumor nodules in the liver were strongly labeled with antibodies against β-catenin. Furthermore, the subcellular distribution of β-catenin protein in HB differed from that in normal liver: instead of being localized along the cytoplasmic membrane, β-catenin strongly accumulated in the cytoplasm and in the nucleus. Nuclear accumulation may contribute to its oncogenic potential by increasing the transcriptional activity.

A stabilized β-catenin may also contribute to pathogenesis of hepatocellular carcinomas in adults because, in these tumors recently, similar mutations have been found, although at a lower frequency (19–23%; Refs. 26 and 29). Large deletions of *β-catenin* were identified in one sample of HCC from a child, as well as in the cell line HepG2 derived from a childhood HCC (Table 1). This indicates shared pathogenetic mechanisms in HB and childhood HCC, the latter representing a rare tumor in children.

The high frequency of oncogenic β-catenin mutations in HBs indicates an important role for the *wingless* pathway in its molecular pathogenesis. Uncontrolled activation of this developmental signaling pathway may induce inappropriate proliferation of liver progenitor cells, and thereby may induce HB. Additional studies will show if

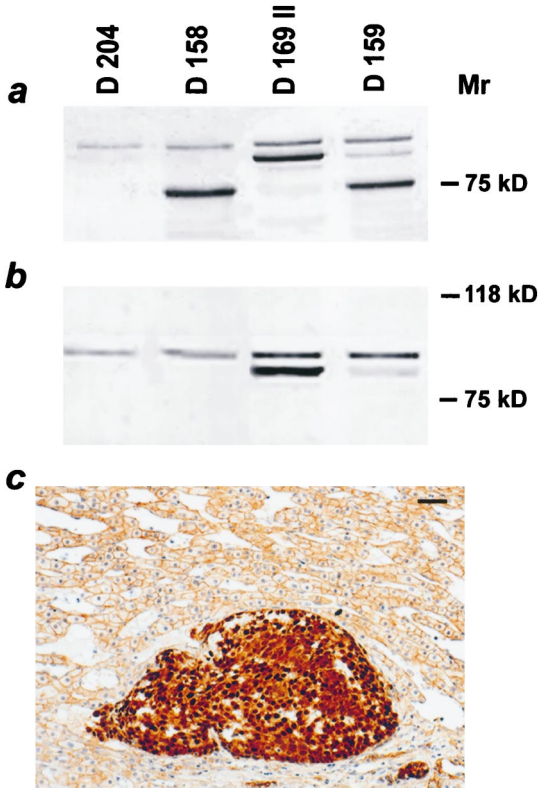

Fig. 4. *a*, Western blot analysis of HBs D204, D158, D169II, and D159 using an antibody that detects wild type and mutant β-catenin proteins. In D204, only the wild-type β-catenin can be detected (this tumor carries a point mutation of β-catenin in exon 3). Tumors D158 and D159 both show an additional band of ~75 kDa, which represents a mutated β-catenin protein with an internal deletion. The mutant β-catenin form in tumor D169II has a smaller deletion. In the latter three tumors, the bands representing the mutated forms are more intense than the band representing the wild type form, indicating an accumulation of the mutated protein. *b*, using an anti-β-catenin antibody recognizing an NH_2-terminal epitope, the location of the internal deletions in the protein degradation box could be verified in tumors D158 and D159 because this antibody is unable to stain the mutated forms of β-catenin. The deletion in tumor D169II is also located in this region, but does not destroy the epitope. *c*, immunohistochemical staining of β-catenin in HB D159 and adjacent normal liver tissue. In normal hepatocytes, β-catenin immunoreactivity is restricted to the cytoplasmic compartment near the cytoplasmic membrane, whereas the nuclei are negative. In contrast, there is marked β-catenin accumulation in both the cytoplasm and the nuclei of the tumor cells. *Bar*, 50 μm.

other molecular components of this pathway are altered and can also lead to HB.

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Childhood Hepatoblastomas Frequently Carry a Mutated Degradation Targeting Box of the β-Catenin Gene

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