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*Advances in Brief*

# Activation of the β-Catenin Gene in Primary Hepatocellular Carcinomas by Somatic Alterations Involving Exon 3[1]

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## Abstract

**We screened 75 primary hepatocellular carcinomas for somatic mutations in the entire coding region of the β-catenin gene. We detected somatic mutations in 14 tumors; 12 were considered to cause amino acid substitutions and 2 were interstitial deletions of 51 or 195 nucleotides of genomic DNA, corresponding to exon 3. Among the 12 point mutations, 6 occurred at potential serine/threonine phosphorylation residues of codons 33, 41, or 45. The remaining six tumors contained a mutation at codon 32 (aspartic acid) or 34 (glycine), flanking to the serine residue at codon 33. By Western blot analysis, we confirmed accumulation of β-catenin in five tumors for which frozen tissues were available; the five included tumors in which amino acid alterations had occurred at codons 32, 34, or 45, and one with a 17-amino acid deletion. Our results suggested that accumulation of β-catenin due to amino acid substitutions at potential serine/threonine phosphorylation residues or at their neighboring codons or interstitial deletions involving exon 3 could contribute to hepatocellular carcinogenesis.**

## Introduction

Continuous destruction and regeneration of hepatocytes as a consequence of hepatitis B or C virus infection often results in chronic hepatitis and subsequent liver cirrhosis. Because these infections are widespread in Asian populations and liver cirrhosis is considered a precancerous lesion for HCC,[3] the incidence of HCC in Asian countries is significantly higher than in other parts of the world (1). However, as with other solid tumors, it is presumed that development and progression of liver carcinomas require an accumulation of genetic alterations in hepatic cells, in addition to the damage occasioned by chronic viral infection. Activation of c-*myc* or cyclin D1 by DNA amplification (2, 3) and inactivation of tumor suppressor functions of *p53* or *Rb* genes (4) have, in fact, been observed in some HCCs, but at relatively low frequencies. Therefore, the genetic mechanism of hepatocellular carcinogenesis remains unsolved.

Reduced expression of E-cadherin due to methylations at CpG sites around the promoter region and allelic deletions of the E-cadherin gene itself have been frequently recognized in HCCs (5, 6). Furthermore, differences in growth patterns or in differentiation grades of HCCs can be correlated with altered expression of E-cadherin (7). E-cadherin forms complexes with catenin molecules such as β-catenin; the complexes connect to the actin filament network and also to cytoplasmic proteins such as fodrin. These interactions play important roles in cell migration, cell separation, and formation of cell layers, processes that polarize hepatocytes and shape hepatic plates in the liver (8).

β-Catenin is involved in the Wnt/Wingless signal-transduction pathway (9); oncogenic activation of this protein by amino acid substitutions or interstitial deletions has been noted in colorectal cancer cell lines and melanoma cell lines, as well as in sporadic colorectal cancers (10–14). Intracellular concentrations of β-catenin are mainly regulated by degradation, which is probably initiated by interaction with APC protein and by phosphorylation at serine and/or threonine residues of codons 33, 37, 41, and 45 through the action of GSK-3β (15–17). Mutations of APC or β-catenin result in stabilization of β-catenin and a significant increase of this protein within the cell. Accumulated β-catenin may translocate into the nucleus, where it could serve as a transcriptional factor through binding with high-mobility group box factors of the Tcf-Lef family (18, 19) and stimulate tumor formation. In addition to decreased levels of E-cadherin, strong immunohistochemical staining of β-catenin has been detected in some HCCs (7). These observations have implied a possible association between disruption of the cadherin-catenin system and tumorigenesis in the liver.

Here, we report results of screening of genetic alterations in the β-catenin gene in 75 primary HCCs and discuss a possible role of β-catenin activation in hepatic carcinogenesis.

## Materials and Methods

**Tissue Samples.** Tumors and corresponding noncancerous liver tissues were obtained from patients who underwent hepatectomy at Osaka Medical Center for Cancer and Cardiovascular Diseases (Osaka, Japan) or Municipal Ikeda Hospital (Osaka, Japan) with informed consent. All tumors were diagnosed histopathologically as HCCs.

**Preparation of DNAs and RNAs.** DNAs were extracted from these tumors and corresponding noncancerous liver tissues, according to methods described elsewhere (14). Total RNAs were also isolated from each samples with TRIZOL Reagent (Life Technologies, Inc.) according to the manufacturer's protocol.

**RT-PCR-SSCP.** Three μg of total RNA were reversely transcribed for single-strand cDNA using oligo(dT)$_{15}$ primer and Superscript II (Life Technologies, Inc.). Each single-strand cDNA was used as a template for subsequent PCRs that covered the entire coding regions with 11 segments. Primer sets used for PCR amplifications were as follows: set 1 (1F, 5′-CCATACAACTGTTTTGAAAATCC-3′; and 1R, 5′-TGAGCTCGAGTCATTGCATAC-3′), set 2 (2F, 5′-CAGTCCTTCACTCAAGAACAAG-3′; and 2R, 5′-ATAGTTAATCAAGTTTACAACTGC-3′), set 3 (3F, 5′-GTTTGGCTGAACCATCACAG-3′; and 3R, 5′-GTTATGCAAGGTCCCAGCGG-3′), set 4 (4F, 5′-ATCATGCGTTCTCCTCAGATG-3′; and 4R, 5′-CACCAGCTA-

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[3] The abbreviations used are: HCC, hepatocellular carcinoma; E-cadherin, epithelial cadherin; APC, adenomatous polyposis coli; GSK-3β, glycogen synthase kinase-3β; RT, reverse transcription; SSCP, single-strand conformation polymorphism.

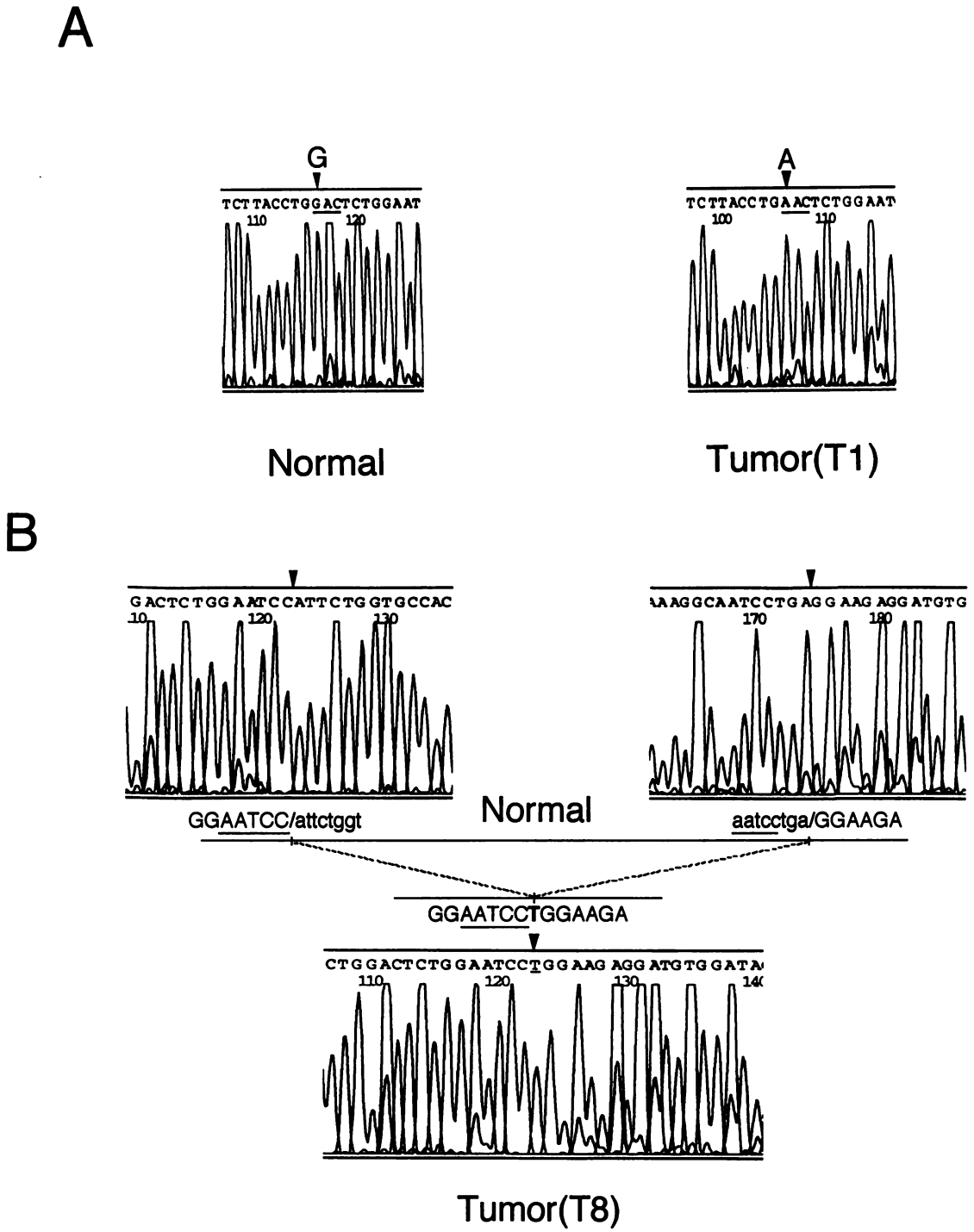


Fig. 1. Sequence analysis of aberrant PCR products of β-catenin found in HCCs. *A*, the normal sequence (*G*) was altered to A in tumor T1, resulting in substitution of AAC (Asn) for GAC (Asp). *B*, a 51-bp interstitial deletion has occurred in tumor T8, with a thymine inserted at the breakpoint instead of adenine. An identical five-nucleotide sequence, AATCC, occurs at both ends of the deletion.

AACGCACTGCC-3′), set 5 (5F, 5′-GCCATTACAACTCTCCACAAC-3′; and 5R, 5′-GATAGCACCTTCAGCACTCTG-3′; set 6 (6F, 5′-TCATACTG-GCTAGTGGTGGAC-3′; and 6R, 5′-CCAAGGAGACCTTCCATCCC-3′), set 7 (7F, 5′-AGATGCTGCAACTAAACAGG-3′; and 7R, 5′-GTGAAGGC-GAACTGCATTCTG-3′), set 8 (8F, 5′-GGTGGTATAGAGGCTCTTGTG-3′; and 8R, 5′-CTGAACTAGTCGTGGAATGGC-3′), set 9 (9F, 5′-ATCAT-GCACCTTTGCGTGAG-3′; and 9R, 5′-TTCAATGGGAGAATAAA-GCAGC-3′), set 10 (10F, 5′-ATGTTCACAACCGAATTGTTATC-3′; and 10R, 5′-GTCAGCTCAACTGAAAGCCG-3′), and set 11 (11F, 5′-TGTTC-CGAATGTCTGAGGAC-3′; and 11R, 5′-GTTCAGAACATACAGCTA-AAGG-3′). The PCR was carried out in a volume of 20 μl for 4 min at 94°C for initial denaturing, followed by 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, on the GeneAmp PCR system 9600 (Perkin-Elmer Corp.). Each 0.2 μg of PCR product was diluted with formamide dye solution and electrophoresed in 5% polyacrylamide gels containing 10% glycerol at 4°C. After electrophoresis, bands were visualized by means of fluorescent image analyzer (FMBIO II Multi-View fluorescent image analyzer; TAKARA) with staining by SYBR Green II (FMC BioProducts).

**Genomic PCR.** We performed PCR amplifications using genomic DNA with a pair of primers, C-F (5′-CCAGCGTGGACAATGGCTAC-3′) and C-R (5′-TGAGCTCGAGTCATTGCATAC-3′), corresponding to parts of DNA sequences of exons 2 and 4, respectively, in the same conditions described

Table 1 *Summary of β-catenin mutations among 75 primary HCCs*

| Tumor | Affected codon(s) | Mutation |
|---|---|---|
| Point mutation | | |
| T1, T2, T11 | 32 | GAC (Asp) → AAC (Asn) |
| T50, TA42 | 32 | GAC (Asp) → TAC (Tyr) |
| T70 | 33 | TCT (Ser) → CCT (Pro) |
| T30 | 34 | GGA (Gly) → GTA (Val) |
| T31 | 41 | ACC (Thr) → GCC (Ala) |
| T65, T67 | 45 | TCT (Ser) → CCT (Pro) |
| T9, TA99 | 45 | TCT (Ser) → TTT (Phe) |
| Interstitial deletion | | |
| T8 | Deletion of 17 amino acids (codons 36–53) | 51-bp deletion (nucleotides 321–372)[a] |
| T32 | Deletion of 50 amino acids (codons 5–54) | 195-bp deletion |

[a] On the basis of the sequence data of GenBank accession no. X87838.

above. The PCR products were electrophoresed in a 1.5% agarose gel and visualized by staining with ethidium bromide.

**Cloning and Sequence Analyses.** Aberrant PCR products detected by RT-PCR-SSCP or genomic PCR were recovered from gels and subcloned into pBluescript II SK(−) vector (Stratagene). The DNA sequences of each clone were determined using an Applied Biosystems model 373 or 377 DNA sequencer (Perkin-Elmer Corp.) with T3 or T7 primers and a Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems). All mutations were confirmed by repeated experiments and Southern hybridization with oligonucleotides corresponding to mutated DNA sequences.

**Western Blot Analysis for β-Catenin.** Extraction of proteins from cancer tissues and following Western blot analyses were performed as described previously (14). Briefly, 20 μg of total protein, extracted from each of the liver cancer tissues T1, T2, T8, T9, and T30, were electrophoresed, transferred to polyvinylidene difluoride membrane, and visualized with anti-β-catenin monoclonal antibody (C19220; Transduction Laboratories).

## Results and Discussion

A total of 75 primary HCCs were screened for genetic alterations of the β-catenin gene by means of the PCR-SSCP technique for an entire coding region using cDNAs that were reversely transcribed from tumor mRNAs and by PCR amplification of genomic DNA of exons 2–4 that included phosphorylation target residues (serine/threonine) present in exon 3 (10–14). Sequence analyses of aberrant PCR fragments detected by SSCP or genomic PCR amplification confirmed somatic alterations that were likely to activate β-catenin in 14 of the 75 cases. For example, in tumor T1 we identified a missense substitution of AAC (Asn) for GAC (Asp; Fig. 1*A*), and in tumor T8, we detected an interstitial deletion of 51-bp within exon 3 that would delete 17 amino acids from the gene product (Fig. 1*B*). The 14 mutations detected in this study are summarized in Table 1 and Fig. 2. Among them, 12 were point substitutions, at codons 32 ($n = 5$), 33 ($n = 1$), 34 ($n = 1$), 41 ($n = 1$), and 45 ($n = 4$). Interstitial deletions, which were detected in ~3% of sporadic colorectal cancers (14), were found in two of our HCC samples. Tumor T8 had a 51-bp interstitial deletion of a part of exon 3, and tumor T32 was found to have a 195-bp deletion corresponding to 5′ portion of exon 3 and a part of intron 2, as shown in Fig. 2. An identical series of five nucleotides (AATCC) was present at each end of the deleted sequence in tumor T8 (Fig. 1*B*). As expected, the RT-PCR using RNAs derived from those two tumors detected aberrant transcripts that were shorter than the normal transcript. DNA sequencing of these bands disclosed that the transcript in tumor T8 had lost 51 bases in exon 3, corresponding to the genomic interstitial deletion, and that the transcript of T32 caused aberrant splicing, resulting in a 150-base deletion of 5′ portion of exon 3 (data not shown). All (in T32) or three (in T8) of the four potential serine/threonine phosphorylation residues were involved in each deletion (Fig. 2).

Because frozen materials were available for 5 of the 14 tumors in which we found β-catenin mutations, we performed Western analysis and confirmed accumulation of normal-size β-catenin (for T1, T2, T9, and T30) or of a slightly smaller molecule corresponding to the predicted product (in tumor T8; Fig. 3). Amino acid substitutions involving serine or threonine residues in exon 3 of β-catenin, which are essential phosphorylation sites for GSK-3β, have been reported in colorectal cancers and melanomas (10–13). In our study of primary HCCs, in addition to mutations at those residues, we demonstrated six missense substitutions at neighboring sites, specifically, at codons 32 (aspartic acid) and 34 (glycine), flanking the serine at codon 33. Furthermore, by Western blot analyses, we confirmed the accumulation of β-catenin in tumors T1, T2, and T30, in which amino acid substitutions had occurred at codons 32 and 34. Others have reported mutations of β-catenin at codons 32 and 34 in chemically induced colon tumors in rats (20). These three residues, DSG (codons 32–34), highly conserved among species, are consensus motifs of the β-cate-

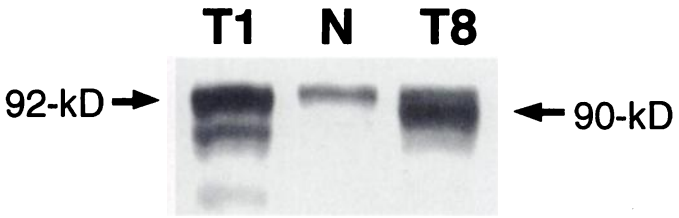


Fig. 3. Western blot analysis of tumors *T1* and *T8* and normal liver (*N*). Accumulation of β-catenin is detected at the normal $M_r$ 92,000 position in T1. In T8, a band of $M_r$ ~90,000 corresponds to the product that lacks 17 amino acids.

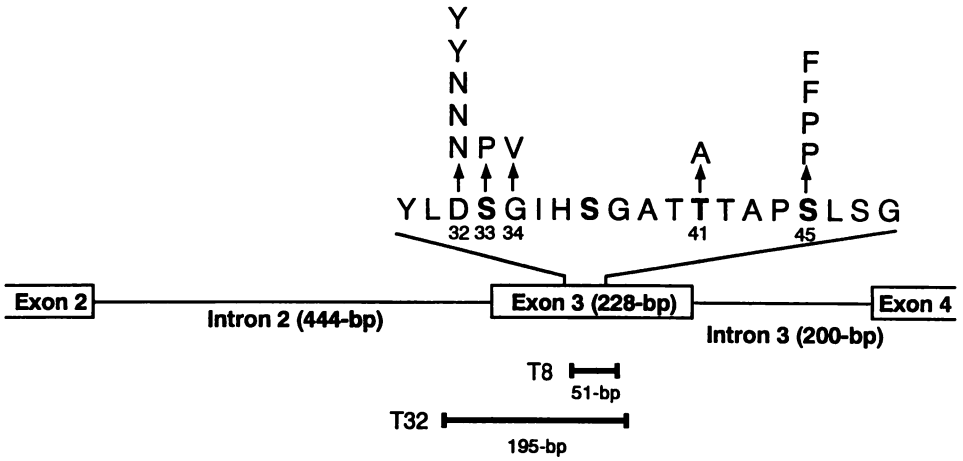


Fig. 2. Schematic representation of 12 amino acid substitutions (5 at codon 32; 1 each at codons 33, 34, and 41; and 4 at codon 45) and two deleted regions (╍, drawn to scale). *Boldface type*, residues representing potential phosphorylation sites.

nin and inhibitor of nuclear factor κB protein families, which are supposed to be necessary for ubiquitin-dependent proteolysis (21). We assume that alterations at codon 32 or 34 may change the protein structure and, thereby, inhibit the phosphorylation of β-catenin or its recognition by the ubiquitin-dependent proteolysis system.

In a previous study (14), we investigated β-catenin mutations in human colorectal cancers and demonstrated a novel β-catenin-activating mechanism, i.e., interstitial deletions involving exon 3. However, unlike other groups (11–13), we found no missense mutations at serine/threonine residues in this exon. We have reported here a novel β-catenin-activating mechanism in human primary HCCs, represented by missense mutations at codons 32 and 34, which does not involve potential phosphorylation sites (serine or threonine residues) in exon 3. Our results suggest that β-catenin accumulation, as a result of amino acid substitutions or interstitial deletions within or including parts of exon 3, can contribute to hepatocellular carcinogenesis. Different types of mutations in the same gene may reflect different etiologies of carcinogenesis in specific tissues, which might reflect dietary or environmental differences or different genetic backgrounds. However, the frequent oncogenic activation of β-catenin in HCCs reported here should contribute to a better understanding of hepatic carcinogenesis.

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