

Frequent Mutations in the β -Catenin Gene in Cholangiocarcinoma

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The molecular pathogenesis of cholangiocarcinoma (CC) remains unclear. β -Catenin functions in both intercellular adhesion and signal transduction. As a signaling molecule, mutations in exon 3 of the β -catenin gene encoding the regions phosphorylated by glycogen synthase kinase (GSK)-3 β stabilize this protein in cytoplasm. Subsequently, accumulated β -catenin protein translocates to nuclei and up-regulates the transcriptional activity of genes involved in oncogenesis. Recently, mutations in exon 3 of the β -catenin gene were detected in various carcinomas. Using polymerase chain reaction (PCR)-single-strand conformational polymorphism (SSCP) analysis, direct sequencing and subcloning-sequencing, we investigated mutations of exon 3 of the β -catenin gene in CC. Mutations were found in 26 out of 33 (78.8%) CC tumor samples. All of the mutations were heterozygous 1-base deletions at codon 15, resulting in a stop codon at codon 46. This is the first study demonstrating the presence of β -catenin gene mutations in CC. However, it was suggested that this mutation might not be involved in deregulation of β -catenin signaling, because no correlation was observed between the β -catenin mutation and immunolocalization of β -catenin protein.

Key words: β -catenin; cholangiocarcinoma; deletion; exon 3; mutation

Cholangiocarcinoma (CC) is an intrahepatic bile duct carcinoma, and is thought to arise from cholangiocytes or liver stem cells (Sell et al., 1989). The molecular mechanism for its carcinogenesis is poorly understood, although some studies demonstrating mutations of *p53* or *K-ras* have been reported in CC (Tada et al., 1992; Tsuda et al., 1992; Kang et al., 1999).

β -Catenin, the vertebrate homologue of armadillo protein in *Drosophila*, is a multifunctional protein involved in cell-cell adhesion regulation coupled with E-cadherin (Ozawa et al., 1989) and the Wnt-Wingless developmental signal-transduction pathway (Gumbiner, 1995). Disruption of this pathway has been suggested in tumorigenesis. In the absence of Wnt signaling, β -catenin is assembled with adenomatous polyposis coli (APC), axin and glycogen synthase kinase (GSK)-3 β and is phosphorylated at exon 3, coding NH₂ terminal serine/threonine

residue by GSK-3 β and is subjected to degradation by the ubiquitin-proteasome system (Aberle et al., 1997). Activation of the Wnt signal pathway inhibits GSK-3 β and subsequently induces β -catenin stabilization. Unphosphorylated β -catenin will not be subjected to ubiquitination and accumulate in cells. β -Catenin translocates to the nucleus with T cell factor (Tcf)/lymphoid enhancer factor (LEF) family members and activates transcription of target genes in cell growth control and apoptosis such as *c-myc* (He et al., 1998) and cyclin D1 (Tetsu and McCormick, 1999). Mutations in the β -catenin gene have been reported in various human tumors and cell lines. The first studies demonstrated mutations at the GSK-3 β phosphorylation site in colon cancers (Morin et al., 1997) and melanoma cell lines (Rubinfeld et al., 1997). In colon cancers, expression of the β -catenin protein in the nucleus increased in

Abbreviations: APC, adenomatous polyposis coli; CC, cholangiocarcinoma; GSK, glycogen synthase kinase; LEF, lymphoid enhancer factor; PCR, polymerase chain reaction; SSCP, single-strand conformational polymorphism; Tcf, T cell factor

tumors with either APC mutations or β -catenin mutations at the GSK-3 β phosphorylation site, because mutations of APC or β -catenin prevented the degradation of β -catenin (Morin et al., 1997; Sparks et al., 1998). Similar mutations in exon 3 of β -catenin have been demonstrated in other cancers and cancer cell lines, including endometrial carcinoma (Kobayashi and Sagae, 1999), thyroid carcinoma (Garcia-Rostan et al., 1999), medulloblastoma (Zurawel et al., 1998), hepatocellular carcinoma (Miyoshi et al., 1998), hepatoblastoma (Koch et al., 1999) and colorectal cell lines (Ilyas et al., 1997). Nuclear and cytoplasmic localization of β -catenin was also reported in endometrial carcinoma (Kobayashi and Sagae, 1999), thyroid carcinoma (Garcia-Rostan et al., 1999), hepatocellular carcinoma (Van Nhieu et al., 1999), and hepatoblastoma (Koch et al., 1999). Therefore it was suggested that mutations in β -catenin were involved in carcinogenesis. However, mutations in the β -catenin gene in CC have never been investigated. The purpose of this study was to examine mutations in exon 3 of the β -catenin gene in CC.

Materials and Methods

DNA preparation

Genomic DNA was extracted from 33 formalin-fixed, paraffin-embedded CC tissues. The age of the 33 patients ranged from 19 years to 81 years with an average of 65 years, and the male to female ratio was 6:5. All tissues of CC were adenocarcinomas, and they were classified into well-differentiated CC (14 patients), moderately-differentiated CC (12 patients) and poorly-differentiated CC (7 patients). DNA was also extracted from peripheral blood of 53 normal Japanese volunteers. The paraffin sections were deparaffinized, and tumor lesions and nontumor liver tissue adjacent to the tumor were scratched up separately under dissecting microscopy (Nikon, Tokyo, Japan). DNA was extracted using standard proteinase K digestion and phenol/chloroform extraction.

PCR/SSCP analysis

Exon 3 of the β -catenin gene was amplified in two polymerase chain reactions (PCRs) using intron-based nested primers. The outside primers for the first reaction were 5'-CCA ATC TAC TAA TGC TAA TAC TG-3' and 5'-CTG CAT TCT GAC TTT CAG TAA GG-3' (Iwao et al., 1998); the inside primers for the second reaction were 5'-TCT ACT AAT GCT AAT ACT GTT TCG-3' and 5'-ATT CTG ACT TTC AGT AAG GCA ATG-3' (Samowitz et al., 1999). The PCR was carried out in a total reaction volume of 20 μ L containing genomic DNA, 10 pmol of each primer, 250 μ mol/L of each dNTP, 1.5 mmol/L MgCl₂ and 0.5 units of Gene *Taq* polymerase (Nippon Gene, Tokyo). The products of the first PCR were used as a template for the second PCR. Both reactions were performed on an ATTO Zymoreactor II (ATTO, Tokyo) with an initial denaturation step at 94°C for 2 min, then 30 cycles at 94°C for 1 min, 58°C for 2 min and 72°C for 2 min, and finally 1 cycle at 72°C for 10 min.

A mixture of 2 μ L PCR products and 5 μ L denaturing solution (98% formamide, 20 mmol/L EDTA and 0.001% bromophenol blue) was heated at 95°C for 5 min followed by rapid cooling on ice and electrophoresed on 14% polyacrylamide gels with 5% glycerol in 1 \times TBE running buffer at room temperature. After electrophoresis, bands were visualized by silver staining (Sugano et al., 1993).

Sequencing

The PCR products with an aberrant band detected by SSCP were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and were subjected to cycle sequencing using the BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin-Elmer Applied Biosystems, Foster City, CA). Sequencing was performed in both directions and each sequencing product was run on an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer Applied Biosystems). Data were analyzed by SequencingAnalysis 3.0 software (Perkin-Elmer Applied Biosystems).

The PCR products with deletion detected by direct sequencing were also subcloned into a pGEM-T Vector (Promega, Madison, WI). The DNA sequence of each clone was determined using an ALFred sequencer (Amersham Pharmacia Biotech, Uppsala, Sweden) with a Cy5 Thermo Sequenase Dye Terminator Kit (Amersham Pharmacia Biotech).

Reliability of the technique

We performed PCR/SSCP analysis repeatedly starting with the first PCR in all CC samples. In addition, to confirm the reliability of our technique, we analyzed the β -catenin gene in thyroid carcinoma ($n = 6$) in which both frozen and formalin-fixed materials were available, using our method. Moreover, we analyzed the β -catenin gene in formalin-fixed colorectal carcinoma ($n = 6$), which was thoroughly studied for β -catenin gene mutation, using our method.

Immunohistochemistry

Immunostaining was performed on 3- μ m paraffin sections with β -catenin monoclonal antibody (clone 14, immunoglobulin G1 class) purchased from Transduction Lab (Lexington, KY) using the antigen retrieval protocol, as previously re-

ported (Ashida et al., 1998). In each β -catenin immunostaining, β -catenin expression was evaluated and the results were semiquantitatively categorized into the seven ranging from +3 to -3 according to the percentage of positive cells within a tumor area in comparison to a nontumor area, as previously reported (Ashida et al., 1998). A nuclear expression was considered positive when more than 10% of nuclei was strongly stained for β -catenin, as previously reported (Terris et al., 1999).

Statistical analysis

Statistical analysis was performed using Fisher's exact test and Mann-Whitney's U test with a significant level of $P < 0.05$.

Results

Exon 3 of the β -catenin gene from CC tumor and nontumor samples was amplified by PCR and the resulting PCR products were screened by SSCP analysis. Representative SSCP gel patterns of 3 tumor and nontumor tissues from CC are shown in Fig. 1. Aberrant bands were detected in the majority of tumor samples. PCR products with aberrant bands detected by SSCP analysis were subjected to direct sequencing. The results of direct sequencing analysis suggested that the β -catenin gene from tumor samples with aberrant SSCP bands might bear heterozygous deletion (Fig. 2a). By subcloning-sequencing, it was confirmed that one allele of the β -catenin gene had a 1-base pair deletion (A) at codon 15 and the other was a wild-type (Fig. 2b). We detected this mutation in 26 of 33 (78.8%) CC tumor tissues. Other types of mutations were not found in this study. This alteration was reproducible by repeated analysis starting with the first PCR. In both the frozen and formalin-fixed thyroid carcinomas used as controls, there were no mutations in any cases. Moreover, we did not detect any mutations in

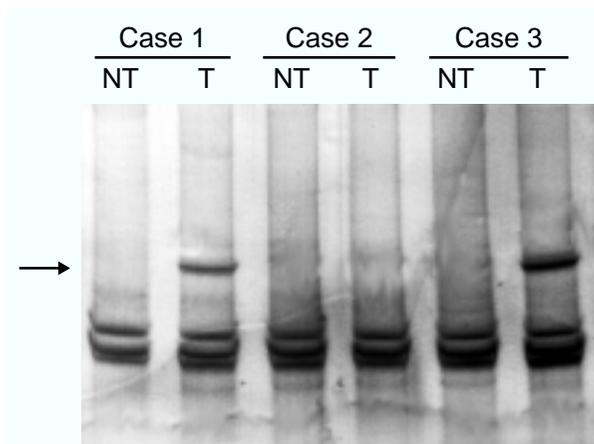


Fig. 1. Single-strand conformational polymorphism analysis of tumor (T) and nontumor (NT) from cholangiocarcinoma samples. Aberrant bands are indicated by the arrow. Mutations were detected in the tumor tissues in cases 1 and 3.

formalin-fixed colorectal carcinoma. This deletion at codon 15 of the β -catenin gene caused a frameshift that resulted in a stop codon 31 amino acids downstream from the mutation, thereby producing a truncated peptide (Fig. 3). Since this mutation was detected in neither

nontumor liver tissue nor peripheral blood from healthy volunteers (Table 1), it was considered that this mutation was somatic and might be related to CC. To the best of our knowledge, this is the first study demonstrating the presence of β -catenin mutations in CC.

Relationships between the β -catenin mutation and the available clinicopathological data were determined (Table 2). No significant difference was found between the β -catenin mutation and the clinicopathological parameters.

Relationships between the β -catenin mutation and immunolocalization were also determined (Table 3). We previously reported that membranous expression was unchanged or decreased in all cases (Ashida et al., 1998). In this study, we reevaluated intracytoplasmic and nuclear expression in CC. Cytoplasmic staining was observed in 8 cases (24%). Among them, 3 cases (9.1%) showed immunoeexpression in the tumor cell nuclei. Membranous expression in cases with mutation did not significantly decrease compared with that in cases without mutation. No correlation was found between β -catenin mutation and immunohistochemical data.

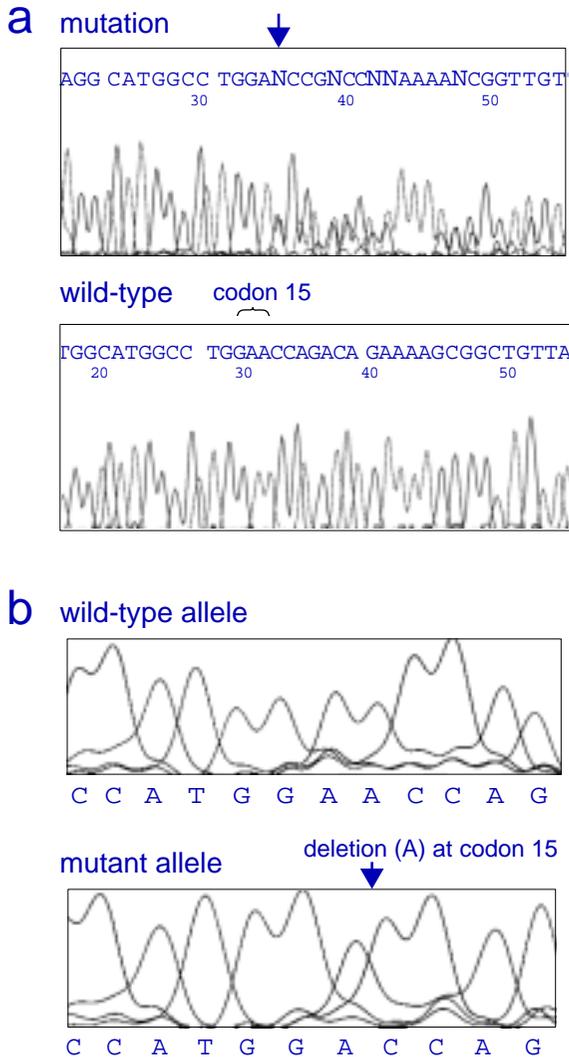


Fig. 2. Sequencing data of β -catenin exon 3 in cholangiocarcinoma (CC) samples.

- a:** Electropherograms by direct sequencing showing the sequence of tumor samples with aberrant bands detected by single-strand conformational polymorphism analysis and wild-type sequence derived from the control sample. The possibility of heterozygous 1-base pair deletion is indicated by the arrow.
- b:** Electropherograms from subcloned polymerase chain reaction products of CC tumor tissues with mutation showing sequences of the wild-type allele and mutant allele. One-base pair (A) deletion was detected at codon 15 in mutant allele.

Mutation of β -catenin in cholangiocarcinoma

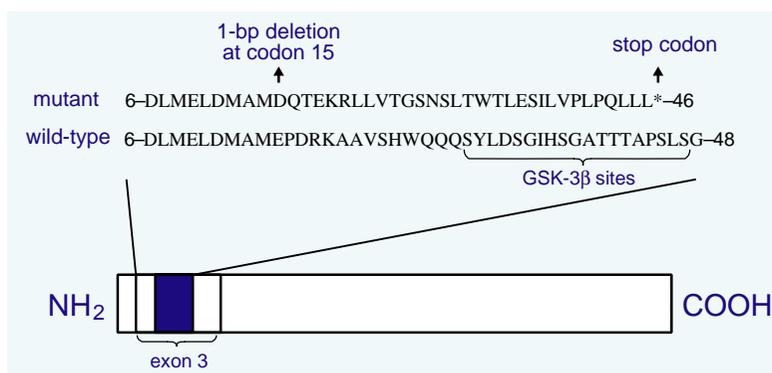


Fig. 3. Schematic representation of the location of β -catenin mutations in cholangiocarcinoma. bp, base pair.

Discussion

The most frequent mutations in various cancers have been shown to result in amino acid substitutions at several serine or threonine phosphorylation sites and flanking residues in the regulatory degradation targeting region encoded by exon 3 (Ilyas et al., 1997; Miyoshi et al., 1998; Garcia-Rostan et al., 1999; Kobayashi and Sagae, 1999; Koch et al., 1999). Small deletions at the GSK-3 β phosphorylation site or larger deletions spanning the whole of exon 3 were also reported in some cancers and all of them were in-frame mutations (Ilyas et al., 1997; Rubinfeld et al., 1997; Iwao et al., 1998; Miyoshi et al., 1998; Koch et al., 1999; Van Nhieu et al., 1999). Therefore it was suggested that unphosphorylated β -catenin accumulated in cytoplasm of cells and translocated to the nuclei. This is consistent with the data of immunohistochem-

Table 1. Alterations in the β -catenin gene

	Frequency of deletion at codon 15 (%)
Tumor tissues (CC)	78.7 [26/33]
Nontumor tissues (CC)	0 [0/33]
Blood (healthy volunteers)	0 [0/53]

CC, cholangiocarcinoma.

ical studies in various carcinomas (Iwao et al., 1998; Garcia-Rostan et al., 1999; Kobayashi and Sagae, 1999; Koch et al., 1999; Van Nhieu et al., 1999). Mutations in most carcinomas were found at a frequency of 10 to 30%, although β -catenin gene alterations in some cancers including thyroid carcinoma (Garcia-Rostan et al., 1999) and hepatoblastoma (Koch et al., 1999) occurred at a frequency of 50 to 60%. However, the mutation detected in this study occurred more frequently (78.8%). Moreover, this

Table 2. Correlations between β -catenin mutation and clinicopathological data

Variable	Category	No mutation	Mutation	P value
Tumor grade	Well-differentiated CC	4	10	NS
	Moderate-differentiated CC	2	10	
	Poor-differentiated CC	1	6	
Vascular invasion	Present	5	22	NS
	Absent	2	4	
Metastasis	Present	5	12	NS
	Absent	2	14	

CC, cholangiocarcinoma; NS, not significant.

Table 3. Relationship between β -catenin mutation and expression of β -catenin protein

	Expression of β -catenin protein									
	Membranous				Cytoplasmic				Nuclear	
	0	-1	-2	-3	0	+1	+2	+3	+	-
No mutation	3	2	2	0	4	2	0	1	1	6
Mutation	11	7	7	1	21	3	2	0	2	24

0, the same expression as that of non-cancerous bile ducts;
 -1, mildly weak expression relative to non-cancerous bile ducts;
 -2, moderately weak expression relative to non-cancerous bile ducts;
 -3, markedly weak expression relative to non-cancerous bile ducts;
 +1, mildly strong expression relative to non-cancerous bile ducts;
 +2, moderately strong expression relative to non-cancerous bile ducts;
 +3, markedly strong expression relative to non-cancerous bile ducts;
 +, nuclear positive expression;
 -, nuclear negative expression.

mutation is a rare frameshift in the coding region, although mutations resulting in a stop codon were also detected in some genes, including E-cadherin in sporadic lobular breast cancer (Berx et al., 1996) and *p53* in ovarian carcinoma (Milner et al., 1993). Although we have used formalin-fixed tissues in this study, it was reported that non-reproducible sequence alterations were detected with the use of formalin-fixed tissues (Williams et al., 1999). This alteration was reproducible by repeated analysis starting with the first PCR. To confirm the reliability of our technique, we analyzed the β -catenin gene in thyroid carcinoma and there were no mutations in both fresh and formalin-fixed specimens. In addition, there were no mutations in the β -catenin gene in formalin-fixed colorectal carcinoma. Therefore, we considered that the technique we used was reliable. Although immunolocalization of β -catenin in CC tissues has hardly been investigated, we previously demonstrated that membranous expression of β -catenin was unchanged or reduced in CC specimens (Ashida et al., 1998). Since immunolabeling was performed using an anti- β -catenin monoclonal antibody, raised against the carboxyl-terminal fragment of β -catenin, it was suggested that normal β -catenin protein expressed in CC tumor tissues. In this study, we demonstrated cytoplasmic and nuclear expression in some cases. However it was considered that aberrant expression of β -catenin protein was not associated with the mutation.

In our study, there were no significant differences between β -catenin mutations and clinicopathologic data. The reason for this is unclear now, and remains to be clarified in the future. It is unclear whether this truncated protein is stable. Commercial antibody against the N-terminal of β -catenin, which is useful for immunohistochemistry, was not available in our study. We also could not perform Western blot analysis because the corresponding frozen tissues were not available. Moreover, we could not make an antibody against the new protein sequence. However, we thought that this protein might be unstable and would immediately degrade, because it is a very small protein consisting of 46 amino acids. It would be interesting to analyze a more related tissue during embryonal development like pancreas carcinoma as a control; however, it was impossible to obtain pancreas carcinoma tissues in the present study. Because no metastatic lesions of CC were available, no investigation of β -catenin in metastatic foci was performed in our study. For these subjects, further studies are required.

It was reported that loss of heterozygosity of APC was detected in CC (23.4%) and the alteration of APC might play a role in the tumorigenesis of CC (Kang et al., 1999). The alteration of APC might be involved in β -catenin protein accumulation because APC interacted with β -catenin in the Wnt signal pathway. In addition, other factors such as axin and GSK- β which were related to the degradation of β -

catenin in the ubiquitin-proteasome system were considered to be associated with increased β -catenin protein expression, since it was recently reported that the mutation in AXIN1, an encoding axin, caused hepatocellular carcinoma (Satoh et al., 2000).

In summary, the present study shows for the first time that somatic mutation in the β -catenin gene occurs frequently in CC. However, no correlation was found between the β -catenin mutation and immunolocalization of β -catenin protein. Therefore, it was suggested that deregulation of β -catenin signaling might not occur in CC. Further investigations are required to elucidate the role of heterozygous mutation in CC.

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