

# $\beta$ -Catenin Mutations in Biliary Tract Cancers: A Population-based Study in China

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## ABSTRACT

$\beta$ -Catenin is an ubiquitously expressed cytoplasmic protein that has a crucial role in both cadherin-mediated cell-cell adhesion and as a downstream signaling molecule in the wingless/Wnt pathway. Activating mutations in exon 3 of the  $\beta$ -catenin gene, at the phosphorylation sites for ubiquitination and degradation of  $\beta$ -catenin, are present in a variety of cancers. Because alterations of the adenomatous polyposis coli (APC) gene are present in biliary tract cancers and the APC protein modulates levels of  $\beta$ -catenin, we evaluated the role of  $\beta$ -catenin in biliary tract cancer by sequencing the third exon of the  $\beta$ -catenin gene among 107 biliary tract cancers and 7 gallbladder adenomas from a population-based study in China. Point mutations of serine or threonine phosphorylation sites in exon 3 of  $\beta$ -catenin were present in 8 of 107 (7.5%) biliary tract cancers and 4 of 7 (57.1%) gallbladder adenomas. Mutations of  $\beta$ -catenin were more frequent in ampullary and gallbladder carcinomas than in bile duct carcinomas ( $P = 0.04$ ) and in papillary adenocarcinomas than other histological types of carcinomas ( $P = 0.02$ ). These results suggest that the molecular pathways of biliary tract neoplasms vary by anatomical subsite and histological subtype.

## INTRODUCTION

Cancers of the extrahepatic biliary tract encompass tumors arising from the gallbladder, extrahepatic bile ducts, and ampulla of Vater. Although most biliary tract cancers are well to moderately differentiated adenocarcinomas, patients generally have a poor prognosis because of spread of tumor to adjacent organs at the time of diagnosis and to the complications associated with surgical resections of the pancreaticobiliary tract (1). Biliary tract cancer is relatively uncommon in most parts of the world, although high-risk populations and upward incidence trends have been reported in certain areas (2, 3).

$\beta$ -Catenin is an intracellular protein that is an integral component of the cadherin-mediated cell-cell adhesion and a downstream transcriptional activator in a Wnt signal transduction pathway (4). The signaling activity of  $\beta$ -catenin is mediated through its interaction with the Tcf/Lef-1<sup>2</sup> family of transcription factors and subsequent activation of target genes (5). Modulation of the level of  $\beta$ -catenin protein is crucial in the regulation of Tcf/ $\beta$ -catenin transcriptional activity, which in turn is regulated through the ubiquitin/proteasome-mediated degradation of  $\beta$ -catenin (6, 7). Activating mutations in exon 3 of the  $\beta$ -catenin gene, at the phosphorylation sites for ubiquitination and degradation of  $\beta$ -catenin, appear to be a crucial step in the progression of a variety of cancers (8). It has been shown that the APC gene product regulates the cytoplasmic level of  $\beta$ -catenin by direct binding to  $\beta$ -catenin and promotes its NH<sub>2</sub>-terminal phosphorylation by GSK-3 $\beta$  (9, 10). GSK-3 $\beta$  phosphorylates multiple serine and threonine residues within the NH<sub>2</sub>-terminal of  $\beta$ -catenin. Phosphorylated

$\beta$ -catenin is targeted for degradation by the proteasome system. Unphosphorylated  $\beta$ -catenin can enter the cell nucleus together with Tcf/Lef-1 and regulates transcription of target genes, including the c-myc signaling pathway (11).

In colon cancer, both activating APC mutations and mutations of the  $\beta$ -catenin gene may result in increased cytoplasmic  $\beta$ -catenin levels with oncogenic activity (8, 12). The role of  $\beta$ -catenin in biliary tract cancer is unclear, but somatic mutations of the APC gene and loss of heterozygosity of chromosome 5q (the location of APC gene) have been reported (13, 14), and ampullary cancers develop in patients with familial adenomatous polyposis (germ-line mutations of the APC gene; Ref. 13).

In this study, we evaluated whether  $\beta$ -catenin mutations are present in sporadic biliary tract carcinomas. To this end, we sequenced exon 3 of the  $\beta$ -catenin gene, which has GSK-3 $\beta$  phosphorylation sites, in 107 biliary tract cancers and 7 gallbladder adenomas collected through a population-based study in Shanghai, China.

## MATERIALS AND METHODS

**Patient Population.** Patients with primary biliary tract cancer (ICD-9 156) newly diagnosed between 1997 and 1999 were identified through a rapid reporting system established between the Shanghai Cancer Institute and 30 collaborating hospitals in urban Shanghai. This reporting system recruited >95% of cases of biliary tract cancers in Shanghai. A total of 107 patients with biliary tract cancers and 7 patients with gallbladder adenomas with severe dysplasia were included in this analysis. These patients were identified as part of a large, ongoing, multidisciplinary, population-based case-control study. Eligibility criteria for case recruitment consisted of residents of urban Shanghai between 18 and 74 years of age diagnosed after April 1997 with gallbladder, extrahepatic bile duct, or ampullary carcinomas.

**Tissue Specimens.** Surgical pathology specimens were collected from patients with biliary tract cancers undergoing curative resection by pancreaticoduodenectomy or bile duct resection or biopsy of an advanced tumor. As part of the case-control study, 6 H&E-stained slides and 6 unstained slides (5  $\mu$ m each) were routinely collected from the surgical pathology departments of the participating hospitals. In addition, a structured questionnaire was used to elicit information on demographic, clinical, and epidemiological variables. The anatomical location of the tumor was recorded in a diagram completed by the local pathologist at the participating hospital. The slides were reviewed by two pathologists from Shanghai and were independently reviewed by one of us (A. R.). The tumors were classified according to the WHO classification of tumors of the biliary tract (15). Medical records were abstracted for all cancer cases. Patients' follow-up was obtained by Shanghai Cancer Institute from the date of diagnosis to May 2000.

**DNA Preparation.** Genomic DNA was extracted from tumor tissue by microdissection from three H&E-stained slides without a coverslip and prepared as described in previous studies (16).

**PCR and Sequencing of  $\beta$ -Catenin.** A 200-bp fragment of exon 3 of  $\beta$ -catenin, encompassing the region of the GSK-3 $\beta$  phosphorylation site, was amplified by PCR as described previously (17). PCR reaction was performed in a 50- $\mu$ l volume using PCR Master (Boehringer Mannheim, Mannheim, Germany) and 1  $\mu$ M of primers (5'-ATGGAACCAGACAGAAAAGC-3' and 5'-GCTACTTGTCTGAGTGAAG-3'), with initial denaturation at 95°C for 5 min; 40 cycles at 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min; and a final extension cycle at 72°C for 10 min (17). The PCR products were treated with shrimp alkaline phosphatase and exonuclease I (Amersham, Buckingham-

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<sup>2</sup> The abbreviations used are: Tcf, T-cell factor; Lef-1, lymphoid-enhancing factor 1; APC, adenomatous polyposis coli; GSK, glycogen synthase kinase.

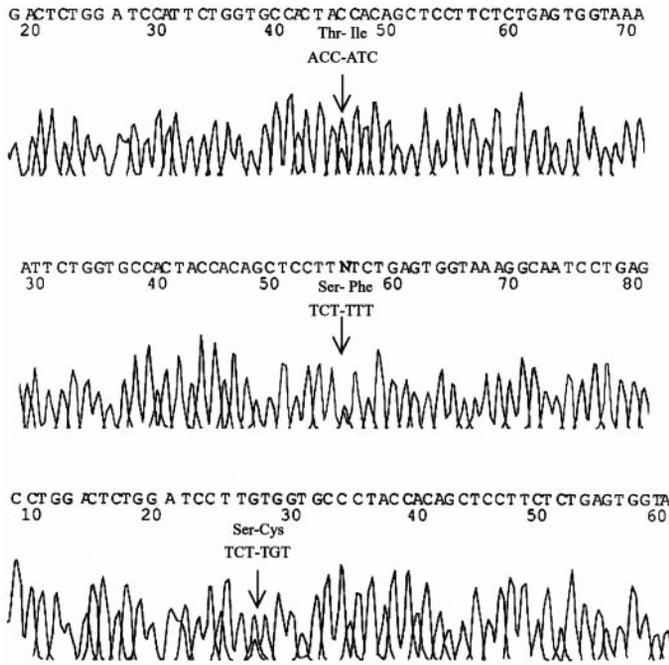


Fig. 1. Nucleotide sequencing of exon 3 of the  $\beta$ -catenin gene in biliary tract carcinomas. The mutations at codons 41, 45, and 33 are indicated by arrows (carcinomas nos. 4–6, Table 1). The wild-type and mutated nucleotide and amino acid sequences are shown on top.

shire, United Kingdom) and sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer, Foster City, CA) with internal primers (5'-AAAGCGGCTGTAGTCACTGG-3' and 5'-GACTTGGGAGG-TATCCACACATCC). The sequence reactions were run on an Applied Biosystems 3700 Genetic Analyzer (Perkin-Elmer, Foster City, CA). The data were collected and analyzed using Applied Biosystems sequencing analysis software, according to the manufacturer's protocols. Each mutation was verified in both directions using the internal primers.

**Statistical Analysis.**  $\chi^2$  or Fisher's exact tests were performed for comparison of frequency of each clinical or pathological characteristic. *t* test was used for comparison of means.

**RESULTS**

Point mutations of the  $\beta$ -catenin gene were present in 8 of 107 (7.5%) invasive biliary tract cancers and 4 of 7 (57.1%) gallbladder adenomas with dysplasia (Fig. 1 and Table 1). All were missense mutations that affected highly conserved serine or threonine important for GSK-3 $\beta$  phosphorylation (Table 1). All four adenomas had missense mutations with replacement of threonine or serine at positions 41 and 45 with isoleucine or phenylalanine. In contrast, point muta-

tions in invasive carcinomas involved replacement of serine or threonine at positions 33, 37, 41, or 45 with cysteine, phenylalanine, alanine, or isoleucine. All four adenomas and three carcinomas had C:G→T:A transition, three carcinomas had C:G→G:C transversion, and two carcinomas had A:T→G:C transition.

The prevalence of  $\beta$ -catenin mutations in invasive cancers varied by anatomical subsite and histological subtype. As shown in Table 2, mutations were present in 2 of 14 (14.3%) ampullary cancers and 6 of 66 (9.1%) gallbladder cancers but in none of 27 bile duct cancers (*P* = 0.04, bile duct versus gallbladder cancers). In addition, mutations occurred in 5 of 24 (20.8%) papillary adenocarcinomas but only in 3 of 68 (4.4%) gland-forming adenocarcinomas not otherwise specified and in 0 of 15 other histological subtypes (*P* = 0.02). The  $\beta$ -catenin mutations found in papillary adenocarcinomas were limited to the gallbladder and were not seen in two papillary carcinomas of the bile duct and ampulla.

Patients whose invasive carcinoma involved a  $\beta$ -catenin mutation had an earlier stage at diagnosis compared with those without mutations (*P* = 0.0007; Table 2). They also had a better prognosis than those whose cancer lacked mutations. Six of 36 patients (16.1%) alive at the end of the follow-up had cancers with  $\beta$ -catenin mutations, whereas 2 of 64 (3.1%) patients who died had mutations (*P* = 0.02). Mean survival time was 21.3 ± 9.9 months for patients with invasive carcinomas with  $\beta$ -catenin mutations versus 11.8 ± 8.5 months for those without mutations. In the subgroup of patients with papillary adenocarcinomas, there was little variation in stage or prognosis by  $\beta$ -catenin mutation status; 16 of 19 (84.2%) without mutation had stage I or II disease at presentation, 13 of 19 (68.4%) were alive at the end of follow-up, and the mean survival was 14.0 ± 7.7 months. In comparison, all six patients with gallbladder adenomas at follow-up were alive after cholecystectomy, irrespective of  $\beta$ -catenin mutation status.

**DISCUSSION**

In this study, we found  $\beta$ -catenin mutations in 14.3% of ampullary cancers, 9.1% of gallbladder cancers, and 57.1% of gallbladder adenomas with severe dysplasia (carcinoma *in situ*). All mutations present in gallbladder adenomas and invasive carcinomas were missense mutations involving highly conserved serine or threonine GSK-3 $\beta$  phosphorylation sites important for  $\beta$ -catenin degradation. The serine or threonine phosphorylation sites are important for  $\beta$ -catenin ubiquitination and degradation, and mutations at these sites could lead to  $\beta$ -catenin accumulation and transduction of oncogenic signals (8, 10, 11). Missense mutations in the serine or threonine residues of the  $\beta$ -catenin gene, which are phosphorylated by GSK-3 $\beta$ , are also present in a wide variety of other tumors (8).

Table 1  $\beta$ -catenin mutations in gallbladder adenomas and biliary tract carcinomas

No.	Site	Histological type	Codon	Mutation	Amino acid change
<b>Biliary tract carcinomas</b>					
1.	Gallbladder	Papillary adenocarcinoma	37	TCT→TGT	Ser→Cys
2.	Gallbladder	Papillary adenocarcinoma	37	TCT→TGT	Ser→Cys
3.	Gallbladder	Papillary adenocarcinoma	41	ACC→GCC	Thr→Ala
4.	Gallbladder	Papillary adenocarcinoma	41	ACC→ATC	Thr→Ile
5.	Gallbladder	Papillary adenocarcinoma	45	TCT→TTC	Ser→Phe
6.	Gallbladder	Adenocarcinoma, NOS <sup>a</sup>	33	TCT→TGT	Ser→Cys
7.	Ampulla	Adenocarcinoma, NOS	41	ACC→GCC	Thr→Ala
8.	Ampulla	Adenocarcinoma, NOS	45	TCT→TTC	Ser→Phe
<b>Gallbladder adenomas</b>					
9.	Gallbladder	Adenoma with severe dysplasia	41	ACC→ATC	Thr→Ile
10.	Gallbladder	Adenoma with severe dysplasia	41	ACC→ATC	Thr→Ile
11.	Gallbladder	Adenoma with severe dysplasia	45	TCT→TTT	Ser→Phe
12.	Gallbladder	Adenoma with severe dysplasia	45	TCT→TTT	Ser→Phe

<sup>a</sup> NOS, not otherwise specified.

Table 2 β-catenin mutation status in invasive biliary tract carcinomas compared with patient demographics and tumor characteristics

	Total No. (%)	β-catenin mutations		P
		Absent No. (%)	Present No. (%)	
Age ± SD (yr)	64.6 ± 8.9	64.2 ± 9.2	68.0 ± 5.9	NS <sup>a</sup>
Gender				
Female	64 (100.0)	57 (89.1)	7 (10.9)	NS
Male	43 (100.0)	42 (97.7)	1 (2.3)	
Tumor site				
Gallbladder	66 (100.0)	60 (90.9)	6 (9.1)	0.04 <sup>b</sup>
Bile duct	27 (100.0)	27 (100.0)	0 (0.0)	
Ampulla	14 (100.0)	12 (85.7)	2 (14.3)	
Histological type				
Papillary adenocarcinoma	24 (100.0)	19 (79.2)	5 (20.8)	0.02
Adenocarcinoma, NOS <sup>c</sup>	68 (100.0)	65 (95.6)	3 (4.4)	
Other histological types <sup>d</sup>	15 (100.0)	15 (100.0)	0 (0.0)	
Stage				
I	18 (100.0)	12 (66.6)	6 (33.3)	0.0007
II	33 (100.0)	32 (97.0)	1 (3.0)	
III	19 (100.0)	18 (94.7)	1 (5.3)	
IV	35 (100.0)	35 (100.0)	0 (0.0)	
Unknown	2 (100.0)	2 (100.0)	0 (0.0)	
Vital status				
Alive	36 (100.0)	30 (83.3)	6 (16.6)	0.02
Dead	64 (100.0)	62 (96.9)	2 (3.1)	
Unknown	7 (100.0)	7 (100.0)	0 (0.0)	
Mean follow-up ± SD (mo)	12.6 ± 8.9	11.8 ± 8.5	21.3 ± 9.9	NA <sup>e</sup>

<sup>a</sup> NS, not significant.

<sup>b</sup> Gallbladder carcinomas versus bile duct carcinomas.

<sup>c</sup> NOS, not otherwise specified.

<sup>d</sup> Includes 1 intestinal adenocarcinoma, 4 mucinous adenocarcinomas, 4 adenosquamous carcinomas, 1 squamous carcinoma, 3 small cell carcinomas, 1 undifferentiated carcinoma, and 1 carcinosarcoma.

<sup>e</sup> NA, not applicable.

It has been reported that β-catenin mutations are more common in carcinomas of certain histological subtypes, such as endometrioid adenocarcinomas of the ovary (17). In our study, β-catenin mutations were more frequent in papillary carcinomas of the gallbladder than in carcinomas of other histological subtypes of the biliary tract. Loss of 5q, the chromosomal location of the APC gene, has been reported in ampullary and extrahepatic bile duct cancers, but APC gene mutations have been observed only in ampullary carcinomas (4, 5). Thus, it appears that mutations of the APC or β-catenin genes are present in a majority of ampullary cancers and a few gallbladder cancers but do not occur in bile duct cancers, suggesting that alterations of the APC/β-catenin pathway are subsite specific.

β-catenin mutations are more frequent in early-stage carcinomas of the uterine endometrium (18). Similarly, in our study patients with invasive carcinomas of the biliary tract and β-catenin mutations had more favorable survival than those without β-catenin mutations, partly attributable to an earlier stage at presentation among patients with mutations and to a better prognosis associated with papillary adenocarcinomas of the gallbladder (19).

In our study, β-catenin mutations were also present in 4 of 7 gallbladder adenomas with severe dysplasia. The role of gallbladder adenomas in the pathogenesis of carcinoma is uncertain (20, 21). Some have argued that adenomas are precursors to invasive carcinomas of gallbladder, because adenoma remnants are occasionally reported with invasive carcinomas (20). Others have suggested that the vast majority of invasive gallbladder carcinomas evolve from flat dysplasia that are difficult to detect clinically (21). Most gallbladder adenomas have K-ras mutations, but they lack p53 mutations or loss of heterozygosity involving chromosomes 5q, 9p, 13q, 17p, or 18q, which often occur with invasive gallbladder carcinomas (21). Frequent presence of flat dysplasia in patients with gallstones (a risk factor for biliary tract cancer), histopathological evidence of progression from dysplasia to carcinoma (22), and similar molecular abnormalities in flat dysplasia and invasive carcinoma suggest that dysplasia is the precursor of most invasive gallbladder carcinomas (20, 21).

Because K-ras and β-catenin mutations are rarely present in invasive carcinomas, it seems likely that gallbladder adenomas only rarely progress to invasive cancer, although pathogenic mechanisms for adenomas and papillary carcinomas of the gallbladder may be similar.

In summary, our study showed that β-catenin mutations are present in ampullary adenocarcinomas and in papillary adenocarcinomas and adenomas of the gallbladder but not in bile duct carcinomas. These findings suggest that the molecular mechanisms involving biliary tract neoplasms vary by anatomical subsite and histological subtype.

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