Jpn. J. Cancer Res. **93**, 1213–1220, November 2002

Analysis of the β-Catenin/T Cell Factor Signaling Pathway in 36 Gastrointestinal and Liver Cancer Cells

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We investigated the frequency and mechanism of β-catenin/T cell factor (Tcf) signaling activation in a panel of 36 human gastrointestinal and liver cancer cell lines. Reporter assay and electrophoretic mobility shift assay revealed that the β-catenin/Tcf signaling was upregulated in 12 of 12 (100%) colorectal, 5 of 8 (68%) gastric, 2 of 7 (29%) hepatic, and none of 9 pancreatic cancer cell lines. The activation of the pathway was mainly due to the mutation of adenomatous polyposis coli (APC) or β-catenin, and Tcf-4 was highly expressed in these cell lines with upregulated signaling. Nuclear β-catenin was observed not only in the signaling-activated cell lines, but also in 14 of 25 (56%) primary gastric cancers, 15 of 20 (75%) colon cancers, 5 of 19 (26%) hepatocellular carcinomas, and none of 13 pancreatic cancers. The presence of signaling-upregulated gastric cancer cell lines with intact APC and β-catenin suggests the involvement of other mechanisms than mutations of APC or β-catenin.

Key words: β-Catenin — APC — GSK-3β — T cell factor — Gastrointestinal cancer

The adenomatous polyposis coli (APC) tumor suppressor gene is mutated in the germ line of individuals with familial adenomatous polyposis coli (FAP), and acquired APC mutations initiate the majority of human colorectal cancers.[1)] The APC protein associates with β-catenin, a cell adhesion protein that is upregulated by the Wnt family proteins. In normal epithelial cells, both components of APC/β-catenin complex are phosphorylated by serine-threonine kinase, glycogen synthase kinase-3β (GSK-3β), which results in β-catenin effectively being degraded via the ubiquitin-proteasome pathway.[2, 3)] Axin and its homolog conductin were shown to make a bridge between GSK-3β and β-catenin, and to promote the phosphorylation and degradation. Mutant APC proteins are unable to form a complex with β-catenin, and they stabilize cytoplasmic β-catenin by preventing its ubiquitination and degradation by the proteasome.[4)] The stabilized β-catenin accumulates in cytoplasm, interacts with T cell factor/lymphoid enhancer factor (Tcf/Lef) family proteins, translocates into the nucleus and directly regulates expression of target genes, such as *c-myc* and *cyclin D1*.[5, 6)] Mutations of the *β-catenin* gene in exon 3, which contains the phosphorylation sites of GSK-3β, were shown to contribute to the accumulation of β-catenin in cytoplasm and nucleus and to the activation of the β-catenin/Tcf signaling pathway in colorectal cancer cells with an intact *APC* gene.[7)] These mechanisms were also reported in various other cancers.[8)]

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We have recently reported that the TGF-β/Smad signaling pathway, which has an important role in carcinogenesis is impaired in colorectal, gastric, and pancreatic, but not hepatic cancer cell lines.[9)] To elucidate the role of other signaling pathway(s) than TGF-β/Smad in the carcinogenesis of gastrointestinal cancers, we systematically investigated the β-catenin/Tcf signaling pathway in human colorectal, gastric, pancreatic, and hepatocellular carcinoma cells.

MATERIALS AND METHODS

Cell lines Thirty-six cell lines were examined: 8 gastric (AGS, AZ-H6c, MKN1, MKN7, MKN28, MKN45, MKN74, and TMK1), 12 colorectal (CaCO2, CaR-1, Colo320, DLD1, HCT15, HCT116, LOVO, LS174T, RCM1, SW48, SW620, and SW837), 9 pancreas (AsPC1, BxPC3, Hs766T, KP-1N, KP-2N, KP-3N, MIA PaCa-2, PANC-1, and Su.86.86), and 7 hepatic (HepG2, HLE, HLF, HuH6, HuH7, PLC/PRF/5, and SK-Hep-1). Pancreatic cancer cell lines KP-1N, KP-2N, and KP-3N were kindly provided by Dr. Akira Kono (National Kyushu Cancer Center, Fukuoka).[10)] Gastric cancer cell line TMK1 was kindly provided by Dr. Eiichi Tahara (Hiroshima University School of Medicine, Hiroshima).[11)] Gastric cancer cell line AZ-H6c was kindly provided by Dr. Kouichi Hirata (Sapporo Medical University School of Medicine, Sapporo).[12)] All other cell lines were purchased from the American Type Culture Collection (Rockville, MD) or the Japanese Cancer Research Resources Bank (Tokyo).

Human T cell line Jurkat was obtained from RIKEN Gene Bank (Tsukuba). The cells were grown in MEM, DMEM, RPMI 1640, McCoy's 5A, or Leivbovitz's L-15 Medium (GIBCO/BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (GIBCO/BRL) at 37°C in a 5% CO_2 atmosphere according to the instructions of the suppliers.

Tumor samples Twenty-five gastric cancers (mean age, 65.8 years old; 16 males and 9 females), 20 colorectal cancers (mean age, 65.3 years old; 16 males and 4 females), 13 pancreatic cancers (mean age, 60.6 years old; 10 males and 3 females), and 19 hepatocellular carcinomas (mean age, 66.2 years old; 17 males and 2 females) were obtained by surgical resection or at autopsy at Motojima General Hospital and the University of Tokyo Hospital.

Transfection and luciferase assay For the analysis of the APC/β-catenin signaling pathway, cells were seeded into 6-well plates, and transfected 24 h later with 0.38 μg of luciferase reporter construct, pTOPFLASH or pFOP-FLASH, generously provided by Dr. Hans Clevers (University Hospital, Utrecht, The Netherlands), and 0.02 μg of renilla luciferase reporter vector pRL-TK (Promega, Madison, WI) as an internal control using Effectene Transfection Reagent (Qiagen, Hilden, Germany). Cells were harvested 24 h after transfection and dual luciferase assay was performed using the PicaGene dual seapansy system (Toyo Ink, Tokyo). Assays were done in triplicate and performed at least three times.

Immunostaining of β-catenin Cultured cells were seeded onto tissue culture chamber slides and allowed to grow for 36–48 h. The cells were then washed in phosphate-buffered saline (PBS), and fixed in methanol/acetone at −20°C. For the paraffin-embedded tumor specimens, the sections were deparaffinized in xylene and rehydrated in graded ethanol. The endogenous peroxidase activity of the cells was quenched by incubating the cells in 0.3% hydrogen peroxide for 20 min at room temperature. The cells were blocked in PBS containing 5% skim milk for 1 h, and mouse anti-β-catenin monoclonal antibody (Transduction Laboratories, Lexington, KY) at a dilution of 1:500 was applied to the cells, which were then incubated overnight at room temperature. Then, the cells were incubated with biotinylated goat anti-mouse antibody and ABC reagent (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. For primary tumors, the immunostaining results were evaluated semi-quantitatively by comparing the immunostaining intensity of tumor cells with that of the adjacent non-tumorous cells as described.[13] Nuclear expression was considered positive when more than 10% of nuclei strongly stained for β-catenin.

Electrophoretic mobility shift assay (EMSA) As the optimal and mutant Tcf probe, we used double-stranded oligonucleotides ACTCTGGTACTGGCCCTTTGATCT-TTCTGG and ACTCTGGTACTGGCCCGGGGATCT-TTCTGG, which have been described by Barker *et al.*[14] The probes were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Nuclear extracts of cells were prepared using mini-nuclear extraction methods.[15] The binding reaction was performed at room temperature for 30 min in a 10 μl mixture consisting of 4% glycerol, 1 m*M* $MgCl_2$, 0.5 m*M* EDTA, 0.5 m*M* dithiothreitol, 50 m*M* NaCl, and 0.5 μg of poly(dI-dC). Samples were incubated for 30 min at room temperature before addition of 0.25 μg of anti-Tcf-4 monoclonal antibody (Upstate Biotechnology, Lake Placid, NY) and anti-β-catenin monoclonal antibody (Transduction Laboratories). The samples were then incubated for another 20 min. DNA-protein complexes were loaded onto a chilled 4% nondenaturing acrylamide gel. After electrophoresis, the gel was dried, and autoradiography was performed using an FLA 3000 image analyzer (Fuji Photo Film Co., Ltd., Tokyo).

Northern blot analysis of Tcf family Probes for human Tcf-1 (305 bp), Tcf-3 (240 bp), Tcf-4 (327 bp), and Lef-1 (194 bp) were generated by PCR on the basis of reported sequences (GenBank accession number X59869, X62870, Y11306, and AF198532, respectively). Twenty micrograms of total RNA was electrophoresed on a formamide-agarose gel and transferred to a nylon membrane (Amersham Pharmacia Biotech, Buckinghamshire, England). The probes were labeled with [α-^{32}P]dCTP and hybridized with the membrane for 12 h. After several washes of the membrane, autoradiography was performed using an FLA 3000 image analyzer.

Immunoblot of APC Cell lysates were subjected to standard sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). The membranes were incubated with anti-APC mouse monoclonal antibody (Ab-1, Oncogene Research Products, Cambridge, MA) at a dilution of 1:100. After several washes, the membranes were incubated with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin (Amersham Pharmacia Biotech) at a dilution of 1:1000. After several washes, APC products were detected using an enhanced chemiluminescence detection system (ECL kit; Amersham Pharmacia Biotech).

Protein truncation test PCR amplification was used to introduce the T7 promoter sequence and a mammalian translation initiation sequence in-frame with the unique APC sequence. Exons 1–14 were divided into two overlapping segments containing exons 1–11 and exons 9–14. RT-PCR for these two segments was performed using primers described by Prosser *et al.*[16] APC exon 15 including the mutation cluster region (MCR), was amplified using primers described by Prosser *et al.*[16] and van der Luijt *et al.*[17] Protein truncation test analysis was per-

formed by using a PTT (protein truncation test)-nonradioactive assay kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions.

Analysis of degradation-targeting region of exon 3 of *β-catenin* gene A 1.1 kb fragment of β-catenin, exon 2 through exon 4, was amplified using primers, BCAT-F1 (5′-GGTATTTGAAGTATACCATAG-3′) and BCAT-R1 (5′-CTGGTCCTCGTCATTTAGCA-3′). Fifteen microliters of PCR mixture containing 0.05 μg of extracted DNA and 100 pmol of each primer was prepared, and then we executed 30 cycles of the reaction at 94°C for 45 s, at 55°C for 45 s, and at 72°C at 30 s.[18] PCR products were purified using a QIAquick PCR purification kit (Qiagen) and directly sequenced with a primer, BCAT-F2 (5′-GAACCAGACAGAAAAGCGGC-3′), in an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). If abnormal DNA sequences were detected, confirmation was done by cloning the PCR products into pCR2.1 vector (Invitrogen, Carlsbad, CA) and sequencing.

RESULTS AND DISCUSSION

Upregulation of β-catenin/Tcf signaling pathway in colon, gastric, and hepatic, but not pancreatic cancers We first analyzed the activity of the β-catenin/Tcf signaling with a functional reporter assay. The luciferase activity of pTOPFLASH was 0.6 to 30.8-fold higher than that of pFOPFLASH in 36 cell lines. Among the 36 cell lines examined in the present study, APC or β-catenin mutations which cause the transactivation of β-catenin/Tcf signaling have been reported for 12, CaCO2, Colo320, DLD1, HCT15, HCT116, LOVO, LS174T, SW48, SW620, SW837, HepG2, and HuH6.[19, 20] In these 12 cell lines, the luciferase activity of pTOPFLASH was more than two (2.1 to 30.8) fold higher than that of pFOPFLASH (Table I). Therefore, the cells which showed more than twofold higher transcriptional activity of pTOPFLASH than pFOPFLASH were considered as having upregulation of this signaling. In all 12 colorectal cancer (CRC) cell lines, transcriptional activity of pTOPFLASH was 2.1 to 30.8-fold higher than that of pFOPFLASH (Table I). Out of 8 gastric cancer cell lines, 5 cell lines, MKN28, MKN45, MKN74, AGS, and AZ-H6c, showed higher transcriptional activity (3.4 to 7.8-fold) of pTOPFLASH than pFOPFLASH. Out of 7 hepatocellular carcinoma (HCC) cell lines, 2 cell lines, HepG2 and HuH6, showed transcriptional activation (28.2 and 15.1-fold, respectively) of β-catenin/Tcf signaling. In contrast, none of 9 pancreatic cancer cell lines showed transcriptional activation of this signaling.

Table I. β-Catenin/Tcf-regulated Transcription Activity and Positivity of Nuclear Staining of β-Catenin

Cell line	Fold luciferase activity (TOP/FOP)	β-Catenin staining	Cell line	Fold luciferase activity (TOP/FOP)	β-Catenin staining
Gastric cancer			Pancreatic cancer		
AGS	7.0±0.3[a]	+	AsPC1	0.6±0.1	−
AZ-H6C	3.5±0.4[a]	+	BxPC3	0.6±0.1	−
MKN1	0.9±0.3	ND	Hs766T	1.4±0.3	−
MKN7	0.7±0.4	−	KP-1N	1.0±0.2	ND
MKN28	7.7±2.7[a]	+	KP-2N	1.3±0.2	ND
MKN45	3.4±1.2[a]	+	KP-3N	1.3±0.1	ND
MKN74	7.8±1.4[a]	+	MIA PaCa-2	0.7±0.1	ND
TMK1	1.0±0.1	ND	PANC-1	0.8±0.2	−
Colorectal cancer			Su.86.86	1.0±0.2	−
CaR-1	3.7±0.5[a]	+	Hepatocellular carcinoma		
CaCO2	6.5±2.3[a]	+	HepG2	28.2±17.0[a]	+
Colo320	30.8±24.5[a]	+	HLE	0.8±0.1	−
DLD1	7.0±2.2[a]	+	HLF	0.8±0.2	−
HCT15	5.0±0.7[a]	+	HuH6	15.1±0.7[a]	+
HCT116	3.2±1.2[a]	+	HuH7	1.4±0.4	−
LOVO	4.9±1.6[a]	+	PLC/PRF/5	1.3±0.4	−
LS174T	9.2±2.7[a]	+	SK-Hep-1	0.7±0.2	ND
RCM1	2.6±0.8[a]	+			
SW48	8.9±1.5[a]	+			
SW620	5.8±2.7[a]	+			
SW837	2.1±0.1[a]	+			

a) Luciferase activity of pTOPFLASH, more than twice as high as that of pFOPFLASH, was defined as active.
ND: not determined.

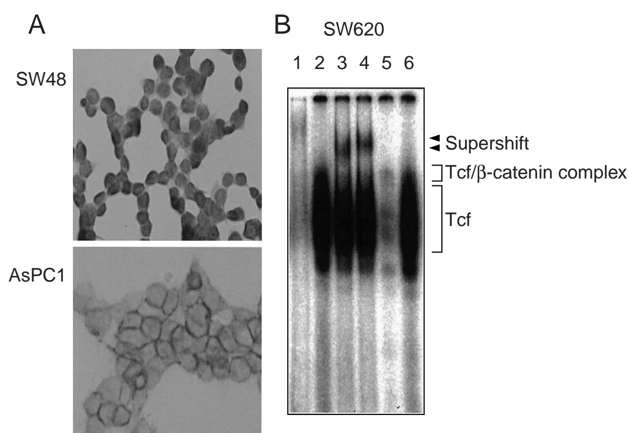

Fig. 1. (A) Immunocytochemical staining of β-catenin. SW48 cells show nuclear staining of β-catenin while membranous β-catenin staining is observed in AsPC1 cells. (B) Electrophoretic mobility shift assay of constitutive β-catenin/Tcf complexes in SW620 cells. The sample in lane 1 was incubated with mutant Tcf. Samples in lanes 2 to 6, were incubated with optimal Tcf probe. Anti-Tcf-4 (0.25 μg) was added to the sample in lane 3. Anti-β-catenin (0.25 μg) was added to the sample in lane 4. Non radiolabeled Tcf probe competitor was added to the sample in lane 5 and mutant Tcf probe was added to the sample in lane 6.

Table II. Expression of mRNA of Tcf Family Members in β-Catenin/Tcf Signaling-activated Cell Lines

Cell line	Tcf-1	Tcf-3	Tcf-4	Lef-1
Colorectal cancer				
CaCO2	+	+	++	+
CaR-1	+	−	++	−
Colo320	+	−	++	++
DLD1	+	+	++	++
HCT15	−	+	++	++
HCT116	+	+	++	−
LOVO	−	+	++	−
LS174T	−	−	++	−
RCM1	++	−	+++	−
SW48	+	−	++	+
SW620	++	+	+++	−
SW837	++	+	++	+++
Gastric cancer				
AGS	++	+	++	−
AZ-H6c	++	−	++	++
MKN28	++	−	++	++
MKN45	−	+	+++	−
MKN74	++	+	++	++
Hepatocellular carcinoma				
HepG2	++	+	++	−
HuH6	++	+	++	++

Expression of mRNA was graded as no expression (−), weak expression (+), moderate expression (++), and strong expression (+++).

In β-catenin/Tcf signaling upregulated cells, β-catenin/Tcf complexes translocate to the nucleus and transactivate downstream target genes, such as *c-myc* and *cyclin D1*.[5, 6] To confirm the data of the reporter assay, localization of β-catenin was assessed by immunocytochemistry and nuclear β-catenin/Tcf complexes were examined by electrophoretic mobility shift assay (EMSA). Nuclear staining of β-catenin was observed in all 12 CRC cell lines, 5 gastric cancer cell lines (MKN28, MKN45, MKN74, AGS, and AZ-H6c) and 2 HCC cell lines (HepG2 and HuH6), all of which showed constitutive upregulation of β-catenin/Tcf signaling by reporter assay. However, in 6 cell lines (MKN7, AsPC1, BxPC3, HLE, HLF, PLC/PRF/5) in which β-catenin/Tcf signaling was not upregulated in the reporter assay, nuclear staining of β-catenin was not observed, but only membranous staining of β-catenin was found (Table I, Fig. 1A). Furthermore, EMSA revealed the presence of nuclear β-catenin/Tcf

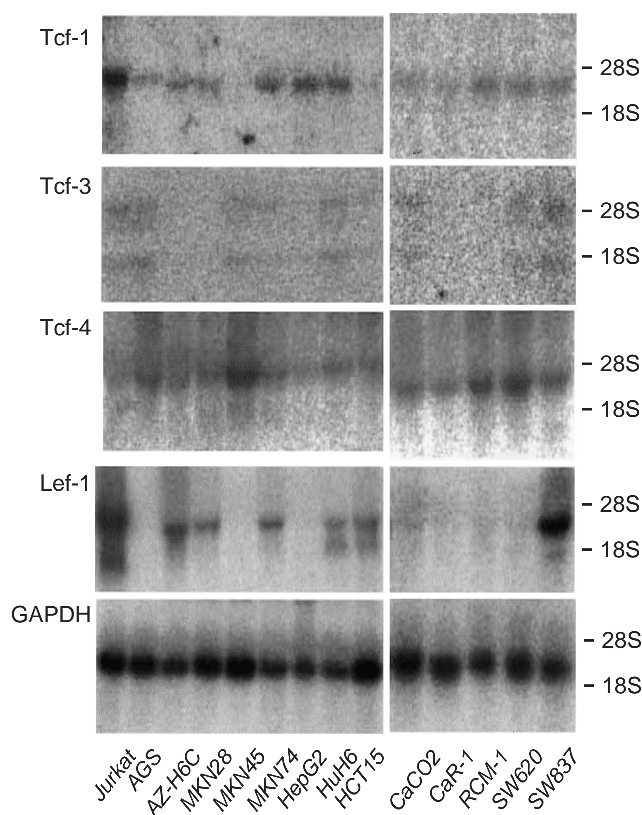

Fig. 2. Northern blot analysis of the Tcf family in β-catenin/Tcf signaling-activated cell lines. Twenty micrograms of total RNA from the indicated cell lines was hybridized to a Tcf-1, Tcf-3, Tcf-4, or Lef-1-specific cDNA probe. The same blot was reprobed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control. Migration of ribosomal RNA 18S and 28S is indicated to the right of panels.

complexes distinguishable by specific retardation of the band with the addition of β-catenin or Tcf antibody in all the β-catenin/Tcf signaling-activated cells as determined by reporter assay (Fig. 1B).

We have recently reported the frequency and mechanism of functional impairment of the TGF-β/Smad signaling pathway in 38 gastrointestinal cancer cell lines.[9] The transforming growth factor (TGF)-β/Smad signaling is impaired in colorectal, gastric, and pancreatic, but not hepatic cancer cell lines,[9] while the β-catenin/Tcf signaling pathway was damaged in colorectal, gastric, and hepatic, but not pancreatic cancer cell lines. Interestingly, in most colorectal and gastric cancer cell lines with upregulated β-catenin/Tcf signaling, the TGF-β/Smad pathway was also impaired. Recently, cross-talk between Wnt and TGF-β signaling was reported.[21] Our studies suggest that functional impairment of both pathways might be important for developing cancer in gastrointestinal mucosa.

Expression of Tcf family in β-catenin/Tcf signaling upregulated cells Four subtypes of the Tcf family, Tcf-1, Tcf-3, Tcf-4, and Lef-1 have been implicated in human cancers.[4, 22, 23] To clarify which Tcf family transcription factors are responsible for the transcriptional activity of β-catenin/Tcf signaling in the cell lines examined, the expression of these four transcription factors was examined by northern blot analysis. Tcf-4 was moderately or strongly expressed in all 19 β-catenin/Tcf signaling-upregulated cell lines (Table II, Fig. 2). However, Tcf-3 expression was weak or not detected in most of these 19 cell lines. On the other hand, Tcf-1 and Lef-1 mRNA expression in these cell lines was different among cell lines, irre-

Table III. Status of APC and Exon 3 of the *β-Catenin* Gene in β-Catenin/Tcf Signaling-activated Cell Lines

Cell line	APC		Exon 3 of β-catenin	
Colorectal cancer				
CaCO2	Mutant	(Mutant)	Wild type	(Wild type)
CaR-1	Mutant		Wild type	
Colo320	Mutant	(Mutant)	Wild type	(Wild type)
DLD1	Mutant	(Mutant)	Wild type	(Wild type)
HCT15	Mutant	(Mutant)	Wild type	
HCT116	Wild type	(Wild type)	ΔS45	(ΔS45)
LOVO	Mutant	(Mutant)	Wild type	(Wild type)
LS174T	Wild type	(Wild type)	S45F	(S45F)
RCM1	Mutant		Wild type	
SW48	Wild type	(Wild type)	S33Y	(S33Y)
SW620	Mutant	(Mutant)	Wild type	(Wild type)
SW837	Mutant	(Mutant)	Wild type	
Gastric cancer				
AGS	Wild type		G34E	
AZ-H6c	Wild type		Wild type	
MKN28	Mutant		Wild type	
MKN45	Wild type		Wild type	
MKN74	Mutant		Wild type	
Hepatocellular carcinoma				
HepG2	Wild type		ΔExon 3	(ΔExon 3)
HuH6	Wild type		G34V	(G34V)

( ): data from the literature (ref. 18, 19).

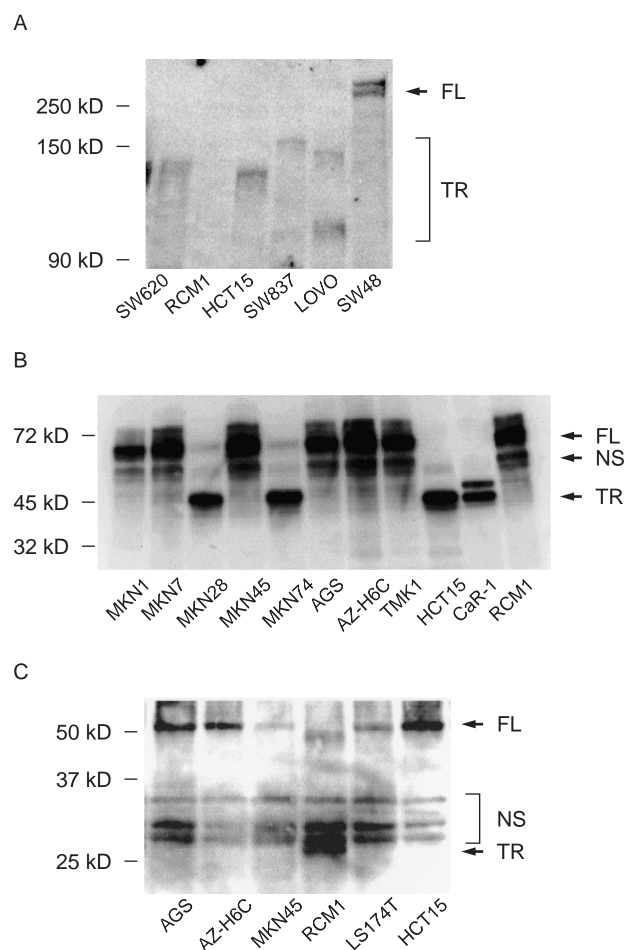

Fig. 3. (A) Immunoblot of APC. Cell lysates were analyzed by 5% SDS-PAGE and blots were probed with anti-APC monoclonal antibody. Truncated products (TR) were observed in SW620, HCT15, SW837, and LOVO cells. Full-length products (FL) were observed in SW48 cells. No APC product was observed in RCM1 cells. (B) Protein truncation test analysis of exon 15 of the *APC* gene. *In vitro* transcription/translation products from the 2-kb T7-PCR products of the APC exon 15 were analyzed by 10% SDS-PAGE. TR were observed in MKN28, MKN74, HCT15, and CaR-1 cells. FL were observed in other cells. NS: non-specific bands. (C) Protein truncation test analysis of exons 1–11 of the *APC* gene. *In vitro* transcription/translation products from the 1.5-kb T7-RT-PCR products of the APC exons 1–11 were analyzed by 10% SDS-PAGE. TR were observed in RCM1 cells. FL were observed in other cells.

spective of the organs of origin. These data suggest that Tcf-4 may be a key isoform in β-catenin/Tcf signaling activation, not only in CRC, but also in gastric cancers and HCC.

Analysis of APC and β-catenin We investigated the status of APC and β-catenin in these cell lines. In CRC cell lines, truncated APC products were detected in CaCO2, Colo320, DLD1, HCT15, LOVO, SW620, and SW837 cells and full-length APC products were observed in HCT116, LS174T, and SW48 cells by immunoblotting and PTT (Table III, Fig. 3, A–C). These results were consistent with previous reports.[19] Only a truncated APC product at the MCR was seen in CaR-1 CRC cells (Fig. 3B). In RCM1 cells, although neither a full-length nor a truncated APC product was detected by immunoblot, a 31-kD truncated APC fragment was detected by PTT of exons 1–11 (Fig. 3C). Mutations of exon 3 of the *β-catenin* gene were detected in 3 CRC cell lines, HCT116, LS174T, and SW48, in accordance with previous reports.[19] Several studies using colorectal cancers revealed that APC mutations were found frequently (about 80%), and that β-catenin mutations are found in less than 10%, mainly in those with intact APC.[24, 25] Our data also suggest that upregulation of the β-catenin/Tcf signaling mainly arises from APC or β-catenin mutations in colorectal cancers.

Of the 5 gastric cancer cell lines with activated signaling (AGS, AZ-H6c, MKN28, MKN45, MKN74), truncated APC products were detected in MKN28 and MKN74 cells, while full-length APC products were observed in AGS, AZ-H6c and MKN45 cells (Table III, Fig. 3, B, C). Of these three cell lines, a GGA to GAA change at codon 34 (G34E) in the *β-catenin* gene was detected in AGS. This mutation supposedly reduces the efficiency of ubiquitin/proteasome-mediated degradation of β-catenin, not by affecting the serine residues which are phosphorylated by GSK-3β, but by affecting the GSK-3β phosphorylation motif (DSGϕXS) that is reportedly essential for ubiquitination of β-catenin.[3] Several reports showed that mutations of *APC* gene were infrequent (less than 10%) in gastric cancers,[26, 27] while β-catenin mutations were found in 26.9% of intestinal-type gastric cancers.[28] Our data suggest that the upregulation of β-catenin/Tcf signaling plays a role in tumorigenesis in a considerable portion of gastric cancers regardless of the presence of APC or β-catenin mutations. There may be a common mechanism of tumorigenesis in cancers of colon and stomach.

The mechanism of activation of this pathway in AZ-H6c and MKN45 cells is not clear from the mutational analysis of APC and β-catenin. Degradation of β-catenin is reported to be regulated by other components, such as Axin1 and Axin2 and in fact, mutation of Axins have been reported in colon and hepatocellular carcinomas.[29, 30] The GSK-3β kinase activity of AZ-H6c and MKN45 was not suppressed as compared with that of the other gastric cancer cells by in-gel kinase assay (data not shown), which suggests that mutation of Axins might cause activation of β-catenin/Tcf signaling in these cell lines.

Table IV. Nuclear Expression of β-Catenin in Primary Gastric, Colorectal, Pancreatic, and Hepatocellular Carcinomas

		β-Catenin staining	
		Positive	Negative
Gastric cancer	(*n*=25)	14 (56%)	11 (44%)
Colorectal cancer	(*n*=20)	15 (75%)	5 (25%)
Pancreatic cancer	(*n*=13)	0 (0%)	13 (100%)
Hepatocellular carcinoma	(*n*=19)	5 (26%)	14 (74%)

Nuclear expression was considered positive when more than 10% of nuclei were strongly stained for β-catenin, as described by Terris *et al.*[13]

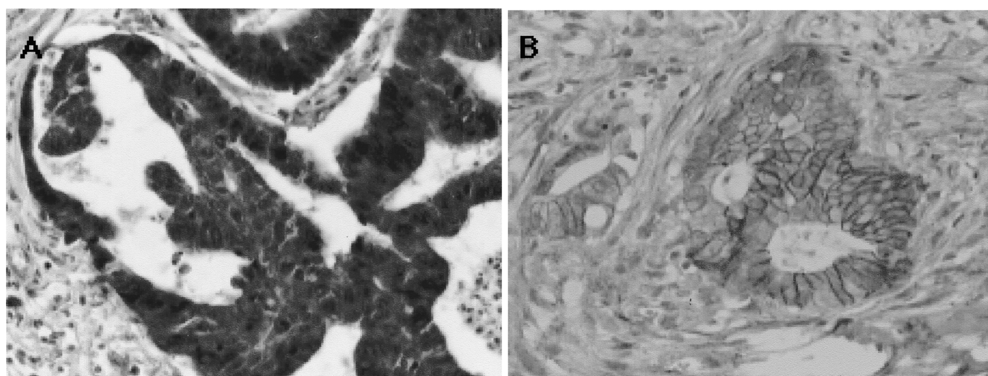

Fig. 4. Immunohistochemical staining of β-catenin in primary tumors. (A) A colon cancer with positive nuclear β-catenin staining. (B) A pancreatic cancer with negative nuclear β-catenin staining.

In the 2 HCC cell lines with activated signaling (HepG2 and HuH6), full-length APC products and mutations of exon 3 of β-catenin were detected, in accordance with previous reports. Mutations of *β-catenin* gene have been reported in about 20% of HCC[20] while mutations of the *APC* gene have rarely been reported. Thus, β-catenin/Tcf signaling may be important for tumorigenesis of a proportion of HCCs, and mutation of the *β-catenin* gene may be a major mechanism for the activation of β-catenin/Tcf signaling in HCC.

Immunohistochemical analysis of β-catenin in primary tumors We performed immunostaining of β-catenin in primary tumors of stomach, colon, pancreas, and liver. Focally or uniformly positive staining of nuclear β-catenin was observed in 14 of 25 (56%) gastric cancers, 15 of 20 (75%) CRC, 5 of 19 (26%) HCC, and none of 13 (0%) pancreatic cancers (Table IV, Fig. 4). These results suggest that β-catenin/Tcf signaling might be also upregulated in primary tumors derived from colon, stomach, and liver. Although APC+/− p53−/− mice were reported to develop pancreatic neoplasia,[31] mutations of *APC* gene appear at low frequency in pancreatic cancers and mutations of β-catenin are rare.[32–34] These results suggest that β-catenin/Tcf signaling may play little role in tumorigenesis of pancreatic cancers.

In conclusion, activation of β-catenin/Tcf signaling may be critical for tumorigenesis in most CRC, and some gastric cancer and HCC. Tcf-4 is the major Tcf family transcription factor which transduces this signaling in colorectal cancer, gastric cancer and hepatocellular carcinomas. The major molecules which transactivate this signaling are different in each cell line. There may be other mechanism(s) than APC or β-catenin mutation underlying some gastric cancers. Furthermore, these data, together with our previous findings on the TGF-β/Smad pathway[9] suggest that there may be some cross-talk between these two signaling pathways in colorectal and gastric carcinogenesis.

ACKNOWLEDGMENTS

We thank Dr. Teiji Motojima, Division of Surgery, Motojima General Hospital, for tumor samples, Dr. Hans Clevers for providing plasmids, and Mitsuko Tsubouchi for technical assistance. This study was supported by the Program for the Organization for Pharmaceutical Safety and Research (OPSR), and the Japanese Ministry of Education, Culture, Sports, Science and Technology (Monbukagakusho).

(Received May 31, 2002/Accepted September 5, 2002)

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