β-Catenin mutations and nuclear accumulation during progression of rat stomach adenocarcinomas

Tetsuya Tsukamoto,[1] Masami Yamamoto,[1] Naotaka Ogasawara,[1] Toshikazu Ushijima,[2] Tomoko Nomoto,[2] Hirofumi Fujita,[3,4] Taijiro Matsushima,[3] Koji Nozaki,[1] Xueyuan Cao[1] and Masae Tatematsu[1]

[1]Division of Oncological Pathology, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya 464-8681; [2]Carcinogenesis Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045; and [3]Japan Bioassay Research Center, 2445 Hirasawa, Hadano-shi, Kanagawa 257-0015

(Received July 31, 2003/Revised October 20, 2003/Accepted October 21, 2003)

Aberrant Wnt/β-catenin signaling caused by mutations in exon 3 of the *β-catenin* gene has been identified in a number of human malignancies, including stomach cancer. However, studies of mutation frequency have yielded conflicting results, and timing during progression remains largely unknown. In this study, we utilized an animal model to address this question. A total of 20 ACI male rats were treated with *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (MNNG) in the drinking water and 22 induced differentiated adenocarcinomas were histopathologically and immunohistochemically evaluated for β-catenin localization. Fourteen tumors (63.6%) that showed homogeneous low-grade morphology, preserving cell polarity, were found to harbor β-catenin protein on the cell membranes (M). Eight tumors exhibited regions of high-grade morphology among areas with low-grade morphology, and they were characterized by denser cell growth and loss of cell polarity. Among these 8 tumors, 4 (18.2%) showed cytoplasmic localization (C) of β-catenin in small regions. The remaining 4 tumors (18.2%) contained more dysplastic regions that displayed nuclear (N) β-catenin staining. Analysis of DNA obtained by microdissection demonstrated that all of 4 regions with C staining and 20 with M staining, as well as 17 samples of surrounding normal mucosa (S) had wild-type β-catenin. In contrast, all of 3 regions with N staining featured mutations (3 of 3=100%; N vs. C, $P<0.05$; N vs. M and N vs. S, $P<0.001$, Fisher's exact test) in exon 3, at glycine 34, threonine 41, and serine 45, which affected phosphorylation sites. In conclusion, *β-catenin* mutations appear to be associated with the late progression stage of adenocarcinoma development in rat stomach carcinogenesis, in contrast to the case of colorectal cancers, in which mutations appear to occur in the early stages. (Cancer Sci 2003; 94: 1046–1051)

Gastric adenocarcinoma is a significant worldwide health burden and is one of the leading causes of cancer deaths. *Helicobacter pylori* infection has recently been implicated as an etiologic factor in gastric cancer development. Gastric cancers exhibit geographical heterogeneity in clinical, biological, and genetic features, and multiple somatic alterations have been described at the molecular level. Studies of loss of heterozygosity (LOH) have shown several chromosomal loci with significant allelic loss, thus indicating that tumor suppressor genes may be important for gastric tumorigenesis.[1] Microsatellite instability has been found to cause gene alteration of the *transforming growth factor β II receptor, insulin like growth factor receptor II, bax, E2F-4, hMSH3*, and *hMSH6* in a subset of gastric carcinomas.[1] The *p53* gene is consistently altered in a majority of gastric cancer cases.[2] Cell adhesion abnormalities such as alterations of E-cadherin or associated molecules caused by gene mutation or by DNA methylation may play an important role in diffuse type gastric cancer development[3] and inactivation of the *adenomatous polyposis coli (APC)* gene has been associated with development of a subset of adenomas and well-differentiated adenocarcinomas.[4] Besides these changes in potential tumor suppressors, reactivation of telomerase, and overexpression of cyclin E have been observed.[4] *Ki-ras* activating mutations and gene amplification and protein overexpression of growth factor receptor *c-erbB2* are preferentially associated with well-differentiated gastric cancers. Conversely, *K-sam* and *c-met* gene amplification is closely linked to poorly differentiated adenocarcinomas.[4]

Molecular analysis has revealed alteration of the APC/Wnt/β-catenin pathway to be involved in many human cancers. *β-Catenin* may harbor mutations in exon 3, where serine and threonine residues are physiologically phosphorylated by glycogen synthase kinase (GSK)-3β. These mutations prevent degradation of β-catenin protein in an APC-dependent manner and cause activation of the β-catenin/Tcf-4 signal transduction pathway.[5] With stomach cancers, although some researchers[6,7] have reported mutations in exon 3 of *β-catenin* gene to be rare, others have observed gene alterations in the range from a few[8–10] to over 20%.[11] However, activation of *β-catenin* has been estimated to occur in 12–37% of cases on the basis of nuclear localization with immunohistochemistry.[7,9,11–13]

Rat stomach neoplasia induced by *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (MNNG) has been widely used as a working model for multistep gastric tumorigenesis of the differentiated type in human stomach.[14,15] The model has been used to identify tumor promoters, to clarify the genetic background to cancer susceptibility, and to examine histological and biochemical changes occurring during stomach carcinogenesis.[16–20] However, there has been little information generated regarding the molecular basis of the process. We previously examined genetic alterations that have been reported in human stomach cancers, using DNA samples extracted from cancers[21] and observed only a few (one in 10 cancers) mutations of the *p53* gene at the second position of codon 171 (Val→Glu) and no mutations in codons 12, 13, or 61 of *Ki-ras* or in the N-terminal phosphorylation sites of the *β-catenin* gene. Furthermore, there was no apparent amplification of the *K-sam* or *c-erbB-2* genes and no microsatellite alterations.

Considering that MNNG-induced rat stomach cancers might allow clarification of the role of *β-catenin* activation during stomach carcinogenesis, we here thoroughly examined β-catenin protein localization by immunohistochemistry and analyzed mutational status using DNA extracted from histologically distinct regions. The results indicated *β-catenin* activation and mutation in small parts of the tumors, suggesting a close link with late stage progression in rat stomach cancers.

Materials and Methods

Experimental design. Twenty male ACI rats at 8 weeks of age were given *ad libitum* drinking water containing 83 mg/l of MNNG (Sigma-Aldrich, St. Louis, MO) in light-shielded bot-

[4]Deceased, May 19, 2002. E-mail: ttsukamt@aichi-cc.jp

tles for 32 weeks and sacrificed at the 72nd experimental week (Fig. 1). Necropsies were performed on all animals that died or were killed when they became moribund. The excised stomachs were fixed in 10% formalin in phosphate-buffered saline, cut into 8 strips, routinely processed, and embedded in paraffin.

Histopathological analysis. Tissue sections were stained with hematoxylin and eosin (H&E) for histological diagnosis. For immunohistochemistry, serial sections were deparaffinized, antigen-retrieved with heating using a microwave oven at 95°C for 10 min in 10 m*M* citrate buffer, pH 6.0, and incubated with monoclonal anti-β-catenin (clone 14, BD Transduction Laboratories, Lexington, KY) antibody at 4°C overnight, followed by application of the avidin-biotin complex method (Vector Laboratories, Inc., Burlingame, CA). The binding was visualized with 3,3′-diaminobenzidine (DAB) (Dojin, Kumamoto) and counter-stained with hematoxylin. Immunoreactivity of β-catenin was classified into "membranous (M)," "cytoplasmic (C)," and "nuclear (N)" according to the intracellular localization of the β-catenin protein. Tumors were then classified into "M" with only membranous β-catenin staining, "C" harboring tumor cells with cytoplasmic β-catenin at least in part, but without nuclear staining, and "N" possessing tumor cells with nuclear accumulation of β-catenin anywhere within the tumor.

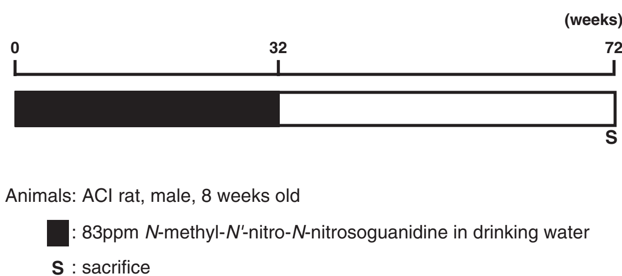

Fig. 1. Experimental protocol. MNNG was administered in the drinking water for 32 weeks.

Microdissection of stomach lesions and polymerase chain reaction-single strand conformation polymorphism analysis (PCR-SSCP). Tumor areas with nuclear, cytoplasmic, or membranous β-catenin localization and surrounding (S) stomach mucosa in serial paraffin sections 10 μm thick were microdissected under a stereoscopic microscope, and genomic DNA was extracted using the Pinpoint Slide DNA Isolation System (Zymo Research, Orange, CA) basically as described earlier.[22] PCR-SSCP analysis of rat β-catenin exon 3 was performed as we detailed previously.[23] Sequences for forward and reverse PCR primers are 5′-GCTGACCTGATGGAGTTGGA-3′ and 5′-GCTACTTGCTCTTGCGTGAA-3′.

Direct sequencing. Sequencing was performed using a BigDye Terminator Cycle Sequencing Kit, v 3.0 (Applied Biosystems, Foster City, CA) with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Results

Histopathological analysis. Stomach lesions were histologically diagnosed basically as described elsewhere[16, 18] (Table 1). Eighteen animals were observed to have 22 differentiated adenocarcinomas. The majority (15 in 22 cases) had preserved cellular and nuclear polarity, as is usually observed for rat stomach adenocarcinomas (Fig. 2A–C, a and b) showing homogeneous low-grade morphology. In contrast, eight lesions showed considerable heterogeneity with high-grade cytological atypia within the tumor masses; some regions of stomach cancers in 4 animals (Tumor Nos. T06-1, T12-1, T12-2, and T17) showed denser proliferation (Fig. 2B, a and d) and another 4 (Tumor Nos. T01, T04, T15, and T20) in part showed large round nuclei and prominent nucleoli, featuring loss of cellular polarity and structural organization (Fig. 2C, a and d).

Table 1. List of rat stomach adenocarcinomas used for mutation analysis of β-catenin

Tumor No.	β-Catenin localization (Sample # for microdissection)				Classification[1)]
	Tumors			Normal surrounding	
	Nuclear	Cytoplasmic	Membranous		
T01	1		2	3	N
T03			20	21	M
T04	4		5	6	N
T05			22	23	M
T06-1		7		9	C
T06-2			8		M
T09			25	26	M
T10-1			28		M
T10-2			29	30	M
T11			32	33	M
T12-1		35	36	37	C
T12-2		38	39		C
T13-1			41		M
T13-2			43	44	M
T14			45	46	M
T15	10		11	12	N
T16-1			47		M
T16-2				49	M
T17		51	52	53	C
T18			54	55	M
T19			13	14	M
T20	15		16	17	N

1) M, tumors with only membranous β-catenin staining; C, tumors with cytoplasmic β-catenin staining at least in part but without nuclear staining; N, tumors with nuclear β-catenin staining at least in part.

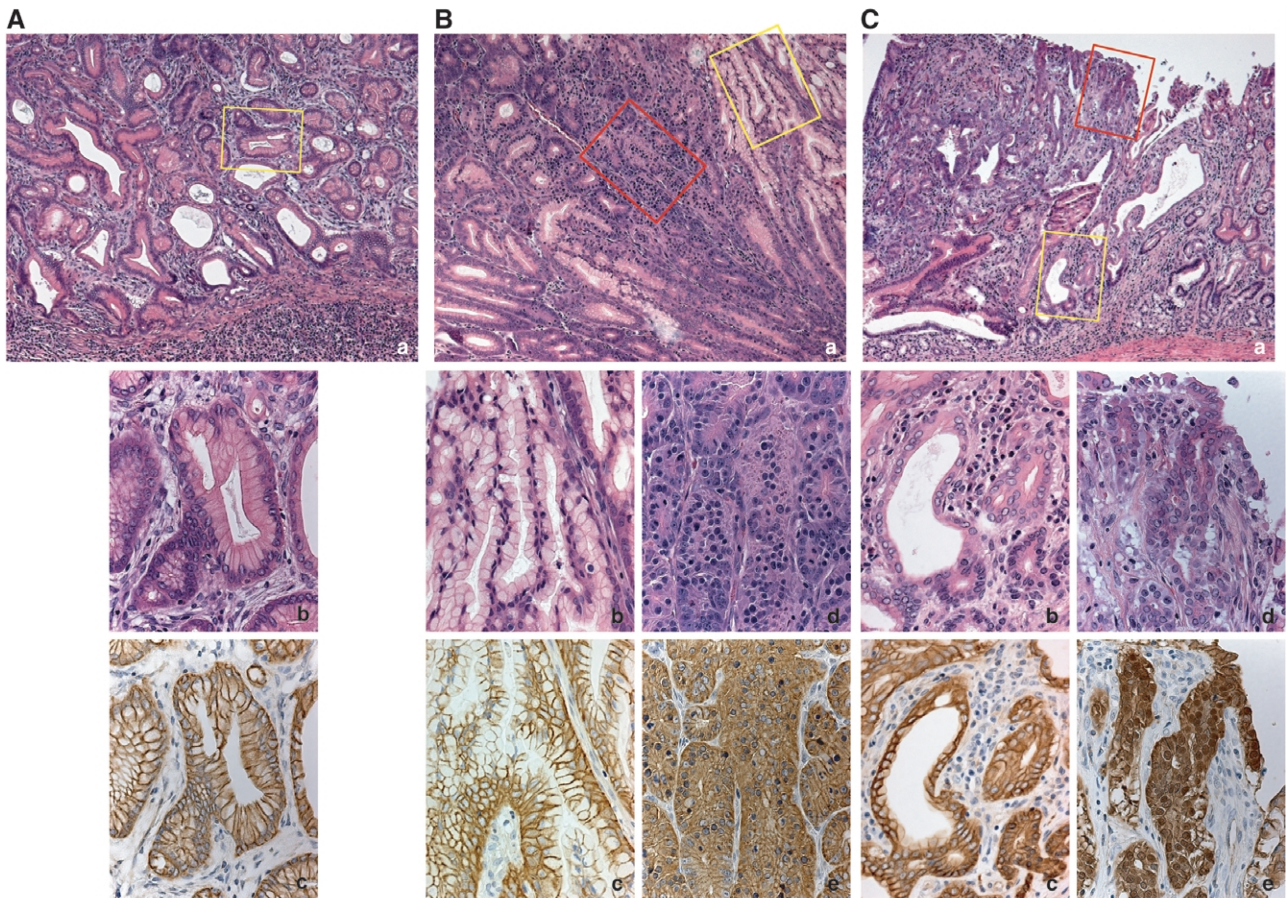

Fig. 2. MNNG-induced rat stomach cancers classified according to β-catenin localization. (A) "M" adenocarcinoma (a) with only membranous β-catenin staining (b and c). (B) "C" carcinoma (a) harboring tumor cells with cytoplasmic β-catenin (d and e) among those with membranous staining (b and c). (C) "N" carcinoma (a) possessing tumor cells with nuclear accumulation of β-catenin (d and e) within those with membranous β-catenin localization (b and c). (a, b, and d) H&E staining. (c and e) β-catenin immunohistochemistry. Original magnification, 80× (Aa and Ca), 100× (Ba), and 400× (b, c, d, and e). Yellow and red boxes in (a) are magnified in (b) and (c), and (d) and (e), respectively, the former being low-grade morphology and the latter high-grade.

Table 2. Localization of β-catenin in stomach tumors and mutation frequency

	Histological grade	β-Catenin localization	Number and frequency of tumors (%)	Frequency of β-catenin mutation (%)
Gastric tumors	High-grade	Nucleus	4/22 (18.2%)	3/3 (100%)[a]
		Cytoplasm	4/22 (18.2%)	0/4 (0%)[b]
	Low-grade	Membrane alone	14/22 (63.6%)	0/20 (0%)[c]
Surrounding normal tissue		Membrane		0/17 (0%)[d]

a: $P<0.05$ vs. b; $P<0.001$ vs. c and d.

β-Catenin localization. Immunostaining of β-catenin revealed that many of the gastric tumors had only membranous localization of β-catenin (14 out of 22 tumors; 63.6%) (Fig. 2A–C, c and Table 2). Among 8 tumors exhibiting high-grade morphology, 4 tumors (4/22; 18.2%) with dense proliferation had regions with cytoplasmic β-catenin staining, as shown in Fig. 2B, e. The last 4 tumors (4/22=18.2%), losing regular gland structure and cell polarity, had regions with nuclear β-catenin accumulation (Fig. 2C, e). Abnormal β-catenin localization in the cytoplasm and nucleus was strongly associated with high-grade morphology.

***β-Catenin* gene mutations in stomach adenocarcinomas.** To analyze *β-catenin* gene mutations in stomach carcinomas with membranous, cytoplasmic, and nuclear β-catenin immunoreactivity, microdissection was performed of corresponding regions from serial paraffin sections. Representative PCR-SSCP results are shown in Fig. 3. DNA samples from membranous (lanes 2, 5, 8, 11, 13, and 16) and cytoplasmic (lane 7) staining regions showed the same DNA mobility as samples from the surrounding normal tissues (lanes 3, 6, 9, 12, 14, and 17) and a wild-type control (lane W). However, examples with nuclear β-catenin staining (lanes 1, 4, and 15) harbored bands (#1a, #1b, #4a, #15a, and #15b) with abnormal mobility. Sequencing analysis (Fig. 4) confirmed them to have the following mutations: #1 (a and b) harbored an ACC (Thr)→ATC (Ile) missense mutation at codon 41. Similarly, #4a had an ACC (Thr)→GCC (Ala) mutation at codon 41. Two mutations of GGA→(Gly)→GAA (Glu) at codon 34 and TCC (Ser)→TTC (Phe) at codon 45 were found in different alleles in #15a and #15b, respectively (Table 3). To evaluate whether the cancer cells harbored two mutations in two different alleles or whether these mutations were derived from different tumor masses in T20 tumor, we

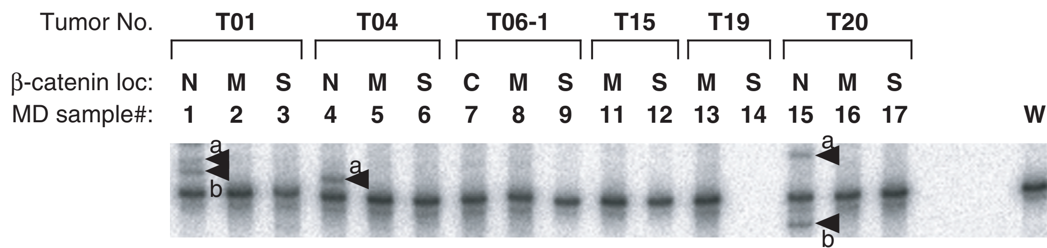

Fig. 3. PCR-SSCP analysis of *β-catenin* exon 3 in rat stomach adenocarcinomas. Only DNA samples with nuclear β-catenin staining (indicated as N) show mobility shifts. Tumor Nos. are listed in Table 1. β-Catenin loc, localization of β-catenin. M, membranous; C, cytoplasmic; N, nuclear; S, surrounding normal mucosa. MD samples #, sample numbers obtained with microdissection (Table 1). W, wild-type control. "a" and "b" indicate abnormal bands.

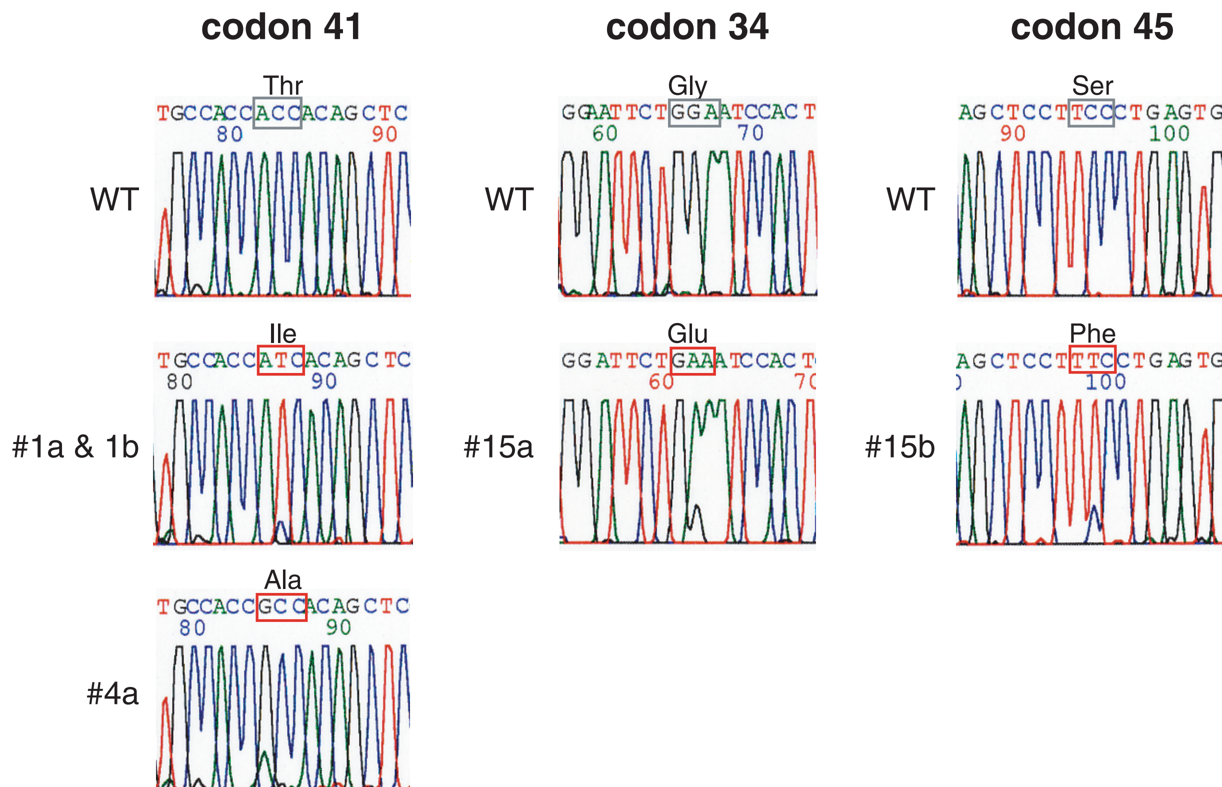

Fig. 4. Sequencing analysis of *β-catenin* gene isolated from rat stomach carcinomas in Fig. 3 showing codons 41, 34, and 45 from the left. The top 3 panels are for the wild type; the middle and the bottom 4 panels are for mutants.

Table 3. Mutation of β-catenin exon 3 in rat stomach tumors

Tumor No.	β-Catenin localization	MD sample #	Band #	Mutation
T01	Nuclear	1	1a	Codon 41: ACC (Thr)→ATC (Ile)
T01	Nuclear	1	1b	Codon 41: ACC (Thr)→ATC (Ile)
T04	Nuclear	4	4a	Codon 41: ACC (Thr)→GCC (Ala)
T20	Nuclear	15	15a	Codon 34: GGA (Gly)→GAA (Glu)
T20	Nuclear	15	15b	Codon 45: TCC (Ser)→TTC (Phe)
	Membrane	W		Wild type

further isolated tissue samples from two topographically different areas. The results suggested that there were two kinds of tumor masses harboring two different β-catenin mutations. The lesion #10 could not be analyzed because of the shortage of the sample material in the paraffin block. In summary, the *β-catenin* gene was mutated at a significantly higher frequency (3 out of 3 tumors=100%) in the stomach carcinomas with nuclear β-catenin staining as compared with other tumors with cytoplasmic ($P<0.05$, Fisher's exact test) or membranous ($P<0.001$) β-catenin staining (Table 2).

Discussion

The present study demonstrated *β-catenin* activation in 18% (4 of 22) of rat MNNG-induced stomach cancers, associated with a subpopulation demonstrating nuclear accumulation of β-cate-

nin within stomach cancer tissue. The results clearly indicate that *β-catenin* activation occurs in the course of stomach cancer progression in this model. In human stomach cancers, there are conflicting reports regarding the incidence and timing of *β-catenin* mutations, although specific occurrence in intestinal-type stomach cancers[24] seems to be a common finding. Both low incidences[6, 9] and relatively high incidences (27–34 %)[11, 12, 24] have been reported, with values tending to be higher in the studies that took advantage of microdissection[9, 11, 24] than in those involving analysis of whole tissue.[6, 7, 10] The findings here for MNNG-induced rat stomach cancers support the notion that *β-catenin* mutations can take place in the course of stomach cancer progression. The apparent discrepancy with our previous report[21] might be due to *β-catenin* activation being confined to a cancer region too small to be detected using DNA extracted from the tumor mass.

In rat models, *β-catenin* mutations have been reported in colon tumors induced by 1,2-dimethylhydrazine,[25] azoxymethane,[26] heterocyclic amines[23, 27, 28] and methylazoxymethanol acetate plus 1-hydroxyanthraquinone.[29, 30] Mutations were found in codons 33, 37, 41, and 45, encoding serine and threonine that are direct targets for phosphorylation by GSK-3β. Also reported were alterations of codons 32, 34, and 35, neighboring serine and threonine residues, possibly resulting in conformational changes and abnormal phosphorylation of the β-catenin protein. In human stomach cancer cases,[9, 11, 24] gene alterations in GSK-3β phophorylation sites in exon 3 included codons 29, 37, 41, and 47. Adjacent sites at codons 28, 32, 34, 39, and 48 also had mutations. In the present MNNG-induced stomach carcinogenesis model, the mutation spectrum was threonine 41 and serine 45 at phosphorylation sites and glycine 34 adjacent to serine 33.

The T20 carcinoma harbored two mutations in different alleles, in codons 34 and 45. Although there was a possibility that the cancer cells had two different mutations in both alleles, direct sequencing of the tissue samples from topographically separate areas revealed that the tumor consisted of heterogeneous populations harboring different mutations. Thus, progression may occur in several regions with different directions from the original low-grade tumor. Tumors with cytoplasmic β-catenin localization did not have any mutations in exon 3 of the *β-catenin* gene. However, the second (exon 5) and third (exon 6) armadillo repeats have been reported to have mutations in human colorectal cancers.[31] Since these regions are important for β-catenin complexing with both APC and E-cadherin, they may be targets. Mutations of not only β-catenin, but also APC cause activation of β-catenin-Tcf signaling in both human[32] and rat colorectal cancers.[27] Thus, mutations of APC in our stomach cancers might explain the observed cytoplasmic β-catenin localization in some cases.

In summary, the morphological progression from low- and intermediate- toward high-grade malignancy appears to be strongly associated with a shift in the localization of β-catenin from the membrane to cytoplasm and nucleus, respectively, coinciding with the *β-catenin* gene mutation status. Activation of the Wnt signaling pathway may thus be an important factor for progression of stomach cancers. This study points to new molecular mechanisms of rat stomach carcinogenesis, and further analysis of oncogenes and tumor suppressor genes, including Wnt signaling molecules, appears warranted.

We thank Ms. Hisayo Ban for expert technical assistance. This work was supported in part by a Grant-in-Aid for the 2nd Term Comprehensive 10-Year Strategy for Cancer Control and a Grant-in-Aid for Cancer Research from the Ministry of Health, Labour and Welfare of Japan and a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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