β-Catenin gene alteration in glandular stomach adenocarcinomas in *N*-methyl-*N*-nitrosourea-treated and *Helicobacter pylori*-infected Mongolian gerbils

Xueyuan Cao,[1,2] Tetsuya Tsukamoto,[1,3] Koji Nozaki,[1,2] Tsutomu Mizoshita,[1] Naotaka Ogasawara,[1] Harunari Tanaka,[1] Yoshiharu Takenaka,[1,2] Michio Kaminishi[2] and Masae Tatematsu[1]

[1]Division of Oncological Pathology, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya, Aichi 464-8681; and [2]Department of Gastrointestinal Surgery, Postgraduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033

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The goal of this study was to elucidate whether *β-catenin* gene mutations might contribute to glandular stomach carcinogenesis in *Helicobacter pylori (H.pylori)*-infected Mongolian gerbils. Firstly, exon 3 of gerbil β-catenin cDNA, a mutation hot spot, was cloned and sequenced and found to have 89.3% homology with the human form and 95.5% with the rat and mouse forms. Peptide sequence in this region was shown to be 100% conserved in these mammals. Then, 45 stomach adenocarcinomas induced with *N*-methyl-*N*-nitrosourea (MNU) plus *H. pylori* infection and 7 induced with MNU alone were examined for β-catenin expression by immunohistochemistry and for DNA mutations using a combination of microdissection and PCR-single strand conformation polymorphism analysis. One gastric cancer in the MNU+*H. pylori* group (2.2%) displayed nuclear (N) β-catenin localization, 3 (6.7%) showed cytoplasmic (C) distribution in local regions, and 41 (91.1%) demonstrated cell membrane (M) localization. Tumors induced by MNU alone showed only membranous β-catenin localization (7/7). Analysis of exon 3 of the *β-catenin* gene demonstrated all tumors with membrane or cytoplasmic staining as well as surrounding normal mucosa (S) to feature wild-type β-catenin. In contrast, the lesion with nuclear staining had a missense mutation at codon 34 [GAC (Gly)→GAA (Glu)] in exon 3 (1/1=100%, N vs. M, $P<0.05$; and N vs. S, $P<0.05$). In conclusion, these results suggest that β-catenin may not be a frequent target for mutation in stomach carcinogenesis in MNU+*H. pylori*-treated gerbils. (Cancer Sci 2004; 95: 487–490)

Abnormal expression of E-cadherin and β-catenin results in loss of epithelial cell-to-cell adhesion, leading to uncontrolled cell growth, and may therefore participate in gastric cancer development.[1,2] However, studies of mutations relevant to Wnt/β-catenin signaling in human stomach tumors have yielded conflicting results.[3–6] We previously reported the existence of *β-catenin* gene mutations in 18% of rat stomach cancers induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG).[7] In contrast, no mutations were found in *N*-methyl-*N*-nitrosourea (MNU)-induced rat gastric carcinomas in another study.[8] Therefore, the clinicopathological significance of *β-catenin* gene mutation is unclear.

Recently, the *Helicobacter pylori* (*H. pylori*)-infected Mongolian gerbil has been established as an appropriate animal model for the study of gastric cancer development, with induction of adenocarcinomas by MNNG or MNU.[9–12] However, little information has thus far been generated regarding molecular events occurring in the gerbil model, partly because of the undefined genetic background.

In this study, stomach adenocarcinomas developing in *H. pylori*-infected or uninfected gerbils treated with MNU in the drinking water were utilized to examine β-catenin protein localization by immunohistochemistry and the mutational status of exon 3 of *β-catenin* gene by using DNA extracted from histologically distinct regions.

Materials and Methods

Tumor samples. Fifty-two gastric adenocarcinomas were collected from 50 gerbils treated with one of three experimental protocols. In experiment I, 28 7-week-old, specific-pathogen-free, male Mongolian gerbils (*Meriones unguiculatus*; MGS/Sea, Seac Yoshitomi, Ltd., Fukuoka) were inoculated with *H. pylori* (ATCC 43504, American Tissue Culture Collection, Rockville, MD), then starting 2 weeks thereafter, were given *ad libitum* drinking water containing 10 ppm of MNU (Sigma Chemical Co., St. Louis, MO) in light-shielded bottles for 20 weeks continuously. In experiment II, 15 gerbils received MNU in water at a concentration of 20 ppm on alternate weeks for a total of 5 weeks exposure and were inoculated with *H. pylori* one week after the completion of this carcinogen exposure. In experiment III, 7 gerbils received MNU only at a concentration of 10 ppm for 20 weeks continuously. All animals were sacrificed at the 70th experimental week. The excised stomachs were fixed in 10% formalin in phosphate buffer for 24 h and samples of tumors and background tissue were routinely processed for embedding in paraffin.

Histopathological analysis. Tissue sections were stained with hematoxylin and eosin (H&E) for histological diagnosis. Immunohistochemical staining with monoclonal anti-β-catenin antibody (clone 14, BD Transduction Laboratories, Lexington, KY) at 4°C overnight followed by the avidin-biotin complex method (Vector Laboratories, Inc., Burlingame, CA) was performed as described earlier.[13] Immunoreactivity of β-catenin was classified into "membranous (M)," "cytoplasmic (C)," and "nuclear (N)" according to the intracellular localization of the protein. Tumors were then classified into "M" with only membranous β-catenin staining, "C" if they harbored tumor cells with cytoplasmic β-catenin at least in part but without nuclear staining, and finally "N" if they possessed tumor cells with nuclear accumulation of β-catenin anywhere within the tumor, as described previously.[7]

Sequencing analysis of β-catenin exon 3. A segment of 224 bp from the genomic DNA in the normal gastric mucosa of Mongolian gerbils was amplified. The PCR product was prepared as the template and the nucleotide sequence was analyzed using a BigDye Terminator Cycle Sequencing Kit, v 3.1 (Applied Biosystems, Foster City, CA) with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Sequences of the forward (5′-GCTGACCTGATGGAGTTGGA-3′) and reverse (5′-GCTACTTGCTCTTGCGTGAA-3′) PCR primers were designed based on the similarity to those of human, mouse, and rat, as described.[13]

PCR-single strand conformation polymorphism (PCR-SSCP) analysis and direct sequencing. Tumor areas and surrounding stomach mucosa were microdissected from 10-μm-thick unstained serial

[3]To whom correspondence should be addressed. E-mail: ttsukamt@aichi-cc.jp

paraffin sections under a stereoscopic microscope, then genomic DNA was extracted using the Pinpoint Slide DNA Isolation System (Zymo Research, Orange, CA) used in our previous work.[7] PCR-SSCP analysis of β-catenin exon 3 was performed with established methods.[13, 14]

Results

β-Catenin localization. Fifty animals were observed to have 45 differentiated and 7 undifferentiated gastric adenocarcinomas. In the MNU+*H. pylori* group, immunostaining of β-catenin revealed that 41 of the demonstrated tumors had only membranous localization (41/45, 91.1%) and 3 had (3/45, 6.7%) cytoplasmic β-catenin staining. The majority of differentiated adenocarcinomas had preserved cellular and nuclear polarity, showing homogeneous low-grade morphology. In contrast, one small lesion showed heterogeneity with high-grade cytological and structural atypia within the tumor masses, where nuclear β-catenin accumulation was observed (1/45, 2.2%, see Fig. 1). On the other hand, all samples (7/7, 100%) in the MNU-only group showed membranous localization (Table 1).

β-Catenin exon 3 sequence of normal gerbil. Sequences of the 224 bp portion of β-catenin exon 3 cDNA in various animals including gerbil, human, rat, mouse, and *Xenopus* were aligned (Fig. 2). The nucleotide sequences of the Mongolian gerbil and human forms exhibited good homology (89.3%), the relation to mouse and rat being even closer (95.5%). Peptide sequences in this region matched completely in the mammals, and almost perfectly (95.9%) with that of *Xenopus*.

***β-Catenin* gene mutations.** Representative PCR-SSCP results are shown in Fig. 3. DNA samples from lesions with membranous and cytoplasmic staining showed similar DNA mobility to that of samples from the surrounding normal tissues and a wild-type control (lane 1). However, the example with nuclear β-catenin staining (lane 2) harbored a band (a) with abnormal mobility. Sequencing analysis confirmed this to be due to a GAC (Gly)→GAA (Glu) missense mutation at codon 34 (Fig. 4).

Discussion

We found only a single mutation at exon 3 of *β-catenin* gene in one of 45 cancers that developed in Mongolian gerbils infected with *H. pylori* and treated with MNU, and 7 cancers in gerbils given MNU alone. To our knowledge, this is the first report of any such mutation of the *β-catenin* gene in a gastric cancer in a Mongolian gerbil. In human and rat lesions, mutations at exon 3 of β-catenin are usually localized at glycogen synthase kinase (GSK)-3β phosphorylation sites (codons 29, 37, 41, and 47) and the adjacent codons (28, 32, 34, 39, and 48), where serine and threonine residues are physiologically phosphorylated. The mutation spectrum of β-catenin in the Mongolian gerbil gastric cancer, although only one was found in this study, was in line with reports on rodent tumors with mutation located at codon 34 of exon 3.[15, 16] Our mutation of *β-catenin* gene was associated with nuclear staining of the protein. We have previously demonstrated such mutations to correlate with nuclear β-catenin in rat gastric cancers induced with MNNG.[7] Ikenoue *et al.*[17] have suggested that the *β-catenin* gene mutation status may be associated with a shift in the localization from the membrane to the nucleus. It is well known that mutations can prevent degradation of β-catenin protein in an APC (adenomatous polyposis coli)-dependent manner and cause activation of the β-

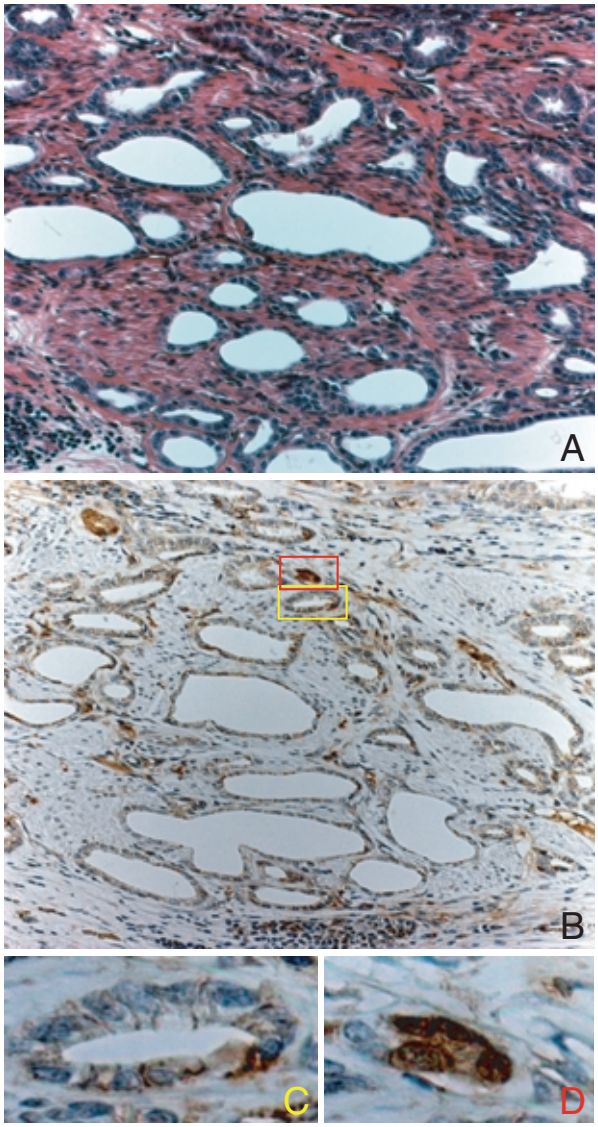

Fig. 1. Mongolian gerbil glandular stomach cancers induced by MNU and *H. pylori* infection. (A) H&E staining of a differentiated adenocarcinoma induced by MNU and *H. pylori* infection (×80). (B) Immunohistochemical analysis of β-catenin protein (×80). (C) Magnified yellow box in (B), representative results for tumor cells with membranous localization of β-catenin (×640). (D) Magnified red box in (B), representative results for tumor cells with nuclear accumulation of β-catenin (×640).

Table 1. β-Catenin localization and exon 3 mutation in Mongolian gerbils' stomach tumors

		β-Catenin localization		β-Catenin mutation	
		MNU+*H. pylori*	MNU only	MNU+*H. pylori*	MNU only
Gastric tumor	Nucleus	1/45 (2.2%)	0/7 (0%)	1/1 (100%)[1)]	0/7 (0%)[4)]
	Cytoplasm	3/45 (6.7%)	0/7 (0%)	0/3 (0%)	0/7 (0%)
	Membrane	41/45 (91.1%)	7/7 (100%)	0/41 (0%)[2)]	0/7 (0%)
Surronding normal tissue	Membrane	45/45 (100%)	7/7 (100%)	0/41 (0%)[3)]	0/7 (0%)

1) $P<0.05$ vs. *2*) and *3*); $P=0.13$ vs. *4*) (Fisher's exact test).

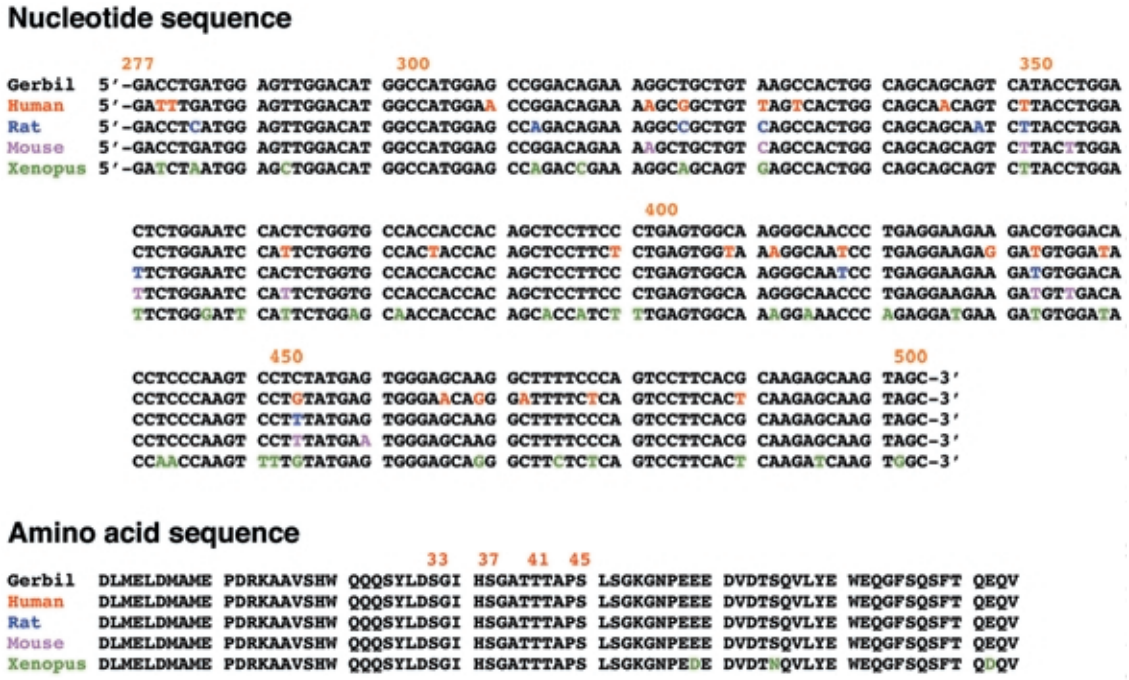

Fig. 2. β-Catenin DNA sequences of Mongolian gerbils in comparison with other species. Top panel, nucleotide sequences of exon 3. Alignment of the 224 bp portion of β-catenin exon 3 cDNA sequences for the Mongolian gerbil, human, rat, mouse, and *Xenopus*: 89.3% oligonucleotide identities were observed between Mongolian gerbil and human, 95.5% with mouse and rat, and 84.4% with *Xenopus*. Bottom panel, the amino acid sequence in exon 3 of β-catenin, which is conserved perfectly among mammals.

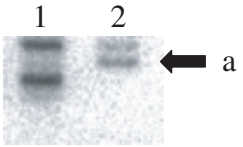

Fig. 3. PCR-SSCP analysis of β-catenin exon 3 in gerbil stomach adenocarcinomas. Lane 1, wild-type control; lane 2, adenocarcinoma sample with nuclear β-catenin staining showing a mobility shift. "a", abnormal band.

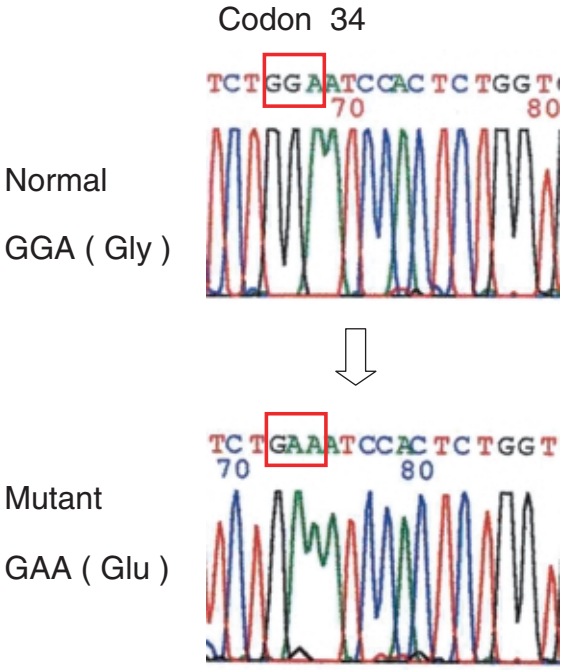

Fig. 4. Sequencing analysis of the *β-catenin* gene isolated from the gerbil stomach carcinoma illustrated in Fig. 3, showing codon 34. Top panel, wild type; bottom panel, mutant.

catenin/Tcf-4 signal transduction pathway in human and rodent models, including rats and mice. Since the sequence of β-catenin exon 3 was highly conserved among the mammals analyzed in this report, the physiological role of β-catenin and the oncogenic mechanism associated with its mutation could be quite similar in Mongolian gerbils as well.

In human stomach cancers, the reported incidences of mutations in exon 3 of *β-catenin* gene have ranged from 0 to over 30%, and loss of E-cadherin expression appears to correlate with poor differentiation and invasion into adjacent organs in adenocarcinomas.[18–21] We have previously revealed that β-catenin mutation occurs in the late stage progression of rat stomach cancers.[7] In addition, Saito *et al.*[22] detected no mutations in exon 3 of the *β-catenin* gene in 9 early-onset human gastric cancers while Clements *et al.*[19] found a significant number of stomach adenocarcinomas with β-catenin mutations and nuclear accumulation, including advanced stage lesions. Therefore, we consider that *β-catenin* gene mutations might be important for late-stage progression in gastric carcinogenesis. β-Catenin activation is usually confined to a small region within a stomach cancer, and thus the use of a microdissection technique to allow sampling of pure populations of tumor cells may prevent false-negative results.[19] The discrepancy in frequency with previous reports could be due to the techniques applied for extraction of DNA from tumor tissues.

In conclusion, mutation of the *β-catenin* gene exon 3 may not be a common event in the generation of stomach cancers in the Mongolian gerbil model with MNU exposure and *H. pylori* infection, but uncontrolled activation of the Wnt signaling pathway could contribute to stomach carcinogenesis in certain tumors. In this study, one β-catenin mutation was detected among

the *H. pylori*-infected gerbils, and there was no statistically significant significance between the MNU+*H. pylori* and MNU-alone groups (1/45 vs. 0/7, $P>0.05$). Thus, *H. pylori* infection may not enhance *β-catenin* gene alteration. It may help clarify the influence of *H. pylori* infection in stomach carcinogenesis to analyze more samples treated with MNU only and to compare the two groups in the future. *H. pylori* infection frequently causes chronic gastritis, and long-term infection increases the risk of gastric cancer. Yu *et al*.[23] earlier found that loss or downregulation of α-catenin mRNA in the gastric mucosa was associated with *H. pylori* infection, which is also known to accelerate E-cadherin methylation.[24] These results are suggestive of activation of the Wnt-catenin-Tcf signaling pathway with *H. pylori* infection in the stomach. β-Catenin was expressed on the membrane of the cancer cells in 48 of 52 (92%) gastric cancer tissues. Thus, other molecular mechanisms, including downregulation of E-cadherin, might have occurred in our model. Whether other genetic or epigenetic alterations occur in gastric cancer cells in cases lacking β-catenin mutations is an intriguing possibility warranting further research.[25, 26]

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