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添付資料：研究報告書

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2．研究テーマ

ヘリコバクターピロリ菌感染スナネズミ胃発がんモデルにおけるbeta-catenin遺伝子変異

3．成果の概要（100字程度）

H. pylori感染＋MNU 投与により発生したスナネズミ腺胃癌組織と周囲の非癌部粘膜について検討した。スナネズミbeta-catenin exon 3 cDNAは、ヒト、ラット、マウスとヌクレオチドレベルでそれぞれ、89.3%、95.5%、95.5% と非常に強い相同性を有した。スナネズミの胃癌では、beta-cateninの遺伝子変異、核内の集積はヒトやラットに比し低頻度であった。しかし、胃癌組織の一部で起こるbeta-cateninの活性化は、種を超えておこる現象と考えられた。

4．研 究 業 績

(1) 学会における発表　☐無　・　☑有（学会名・演題）

第63回日本癌学会学術総会

Helicobacter pylori感染による慢性炎症と胃粘膜細胞増殖がスナネズミ腺胃発癌に影響する

(2) 発表した論文　☐無　・　☑有（雑誌名・題名）

1.Cancer Science　vol 95(6), 487-90
"beta-Catenin gene alteration in glandular stomach adenocarcinomas in N-methyl-N-nitrosourea-treated and Helicobacter pylori-infected Mongolian gerbils"

2.Cancer Science　vol 95(11), 872-7
"Eradication of Helicobacter pylori induces apoptosis and inhibits proliferation of Heterotopic Proliferative Glands in infected Mongolian gerbils"

— 日中医学協会助成事業 —

ヘリコバクターピロリ菌感染スナネズミ胃発がんモデルにおける β-catenin 遺伝子変異

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Abstract

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 beta-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 beta-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.

Key Words Mongolian gerbils, stomach cancer, β-catenin, Helicobacter pylori

Introduction

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 b-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 gerbil model, partly reflecting 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 b-catenin protein localization by immunohistochemistry and the mutational status of exon 3 of b-catenin gene using DNA extracted from histologically distinct regions.

Subjects and methods

Tumor samples

Fifty-two gastric adenocarcinomas were collected from fifty gerbils treated with one of three experimental protocols. In experiment I, twenty-eight 7 week-old male 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 two weeks thereafter, were given drinking water ad libitum containing 10 ppm of MNU (Sigma Chemical Co., St Louis, MO) in light-shielded bottles for 20 weeks continuously. In experiment II, fifteen gerbils received MNU in water at a concentration of 20 ppm for alternate weeks for a total of 5 weeks exposure and 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 24h 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" harboring tumor cells with cytoplasmic β-catenin at least in part but without nuclear staining, and finally "N" possessing tumor cells with nuclear accumulation of β-catenin anywhere within the tumor, as described previously.[7)]

Sequencing analysis of b-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 of 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 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-two animals were observed to have 45 differentiated and 7 undifferentiated gastric adenocarcinomas. In the MNU+H. pylori group, immunostaining of b-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 MNU only group had membranous localization (Table 1).

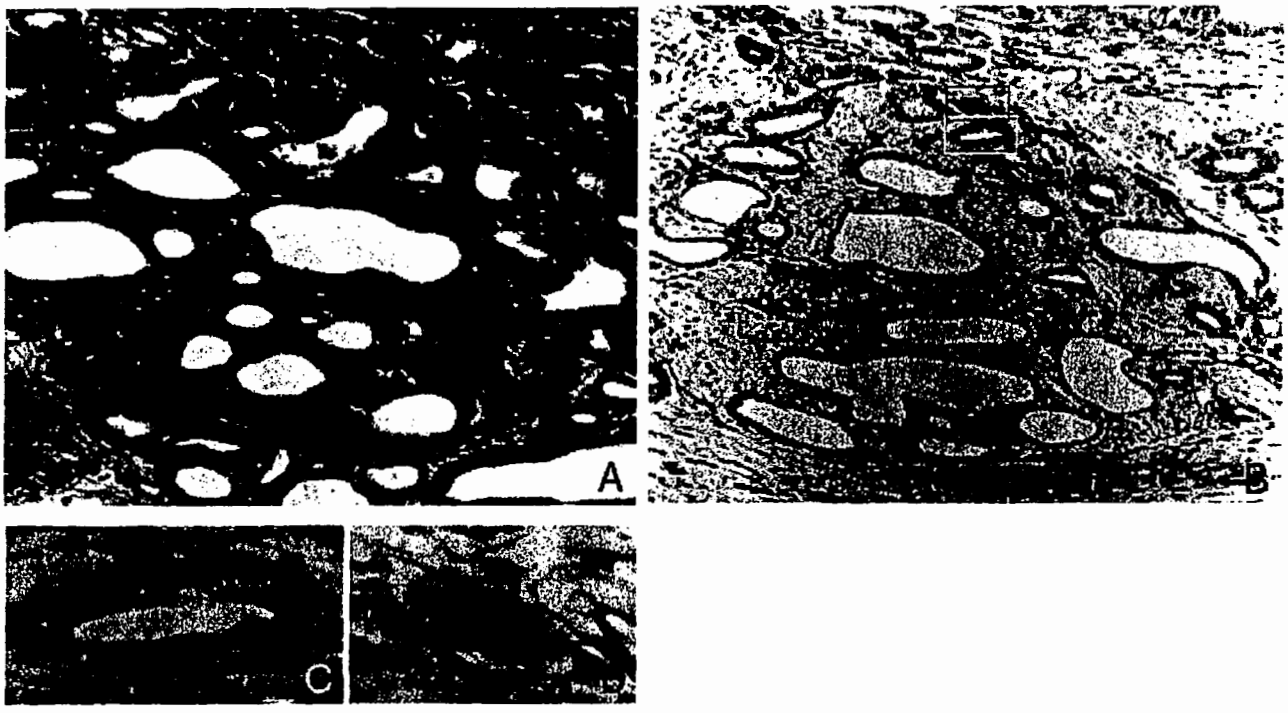

Fig. 1.

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 (X80). (B) Immunohistochemical analysis of β-catenin protein (X80). (C) Magnified yellow box in (B), representative results for tumor cells with membranous localization of β-catenin (X 640). (D) Magnified red box in (B), representative results for tumor cells with nuclear accumulation of β-catenin (X 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%)[a]	0/7 (0%)[d]
	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%)[b]	0/7 (0%)
Surronding normal tissue	Membrane	45/45 (100%)	7/7 (100%)	0/41 (0%)[c]	0/7 (0%)

a: $P < 0.05$ vs.b and c; $P = 0.13$ vs.d. (Fisher's exact test)

β-catenin exon 3 sequence of normal gerbil

Sequences of the 224 bp portion of b-catenin exon 3 cDNA in various animals including a gerbil, human, rat, mouse, and xenopus were aligned (Fig. 2), and the nucleotide sequences of the Mongolian gerbil and human forms found to be demonstrated good homology (89.3%), the relation to the mouse and rat goes being even more close (95.5%). Peptide sequences in this region matched completely in the mammals, and almost perfectly (97.2%) 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 as 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).

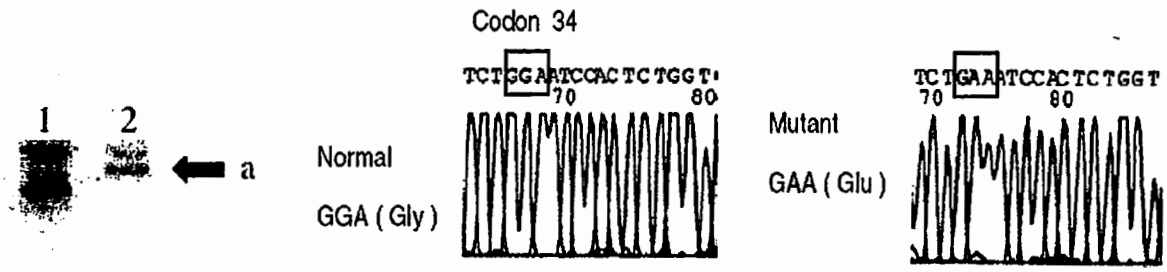

Fig. 3.

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 b-catenin gene isolated from the gerbil stomach carcinoma illustrated in Fig. 3 showing codon 34. Top panel, wild type; bottom panel, mutant.

Discussion

Our present data demonstrated only a single mutation at exon 3 of β-catenin gene observed in one of 45 cancers developing in Mongolian gerbils infected with H. pylori and treated with MNU, and 7 cancers developing in MNU only. 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 case in this study, was in line with the reports for rodent tumors located at codon 34 of exon 3. [15, 16] Our mutation of β-catenin gene was limited to nuclear staining of the protein. We have previously demonstrated such mutations to correlate with the nuclear β-catenin in rat gastric cancers induced with MNNG. [7] Ikenoue et al. [17] have suggested that the β-catenin gene mutation status appears associated with a shift in the localization from the membrane to nucleus. It is well known that the mutations can prevent degradation of β-catenin protein in an APC (adenomatous polyposis coli) dependent manner and cause activation of the β-catenin / Tcf-4 signal transduction pathway in human and rodent models including rats and mice. Since sequences of β-catenin exon 3 were highly conserved among the mammals analyzed in this report, physiological role of β-catenin and oncogenic mechanism associated with its mutation could be quite similar in Mongolian gerbils as well.

In human stomach cancers, the incidences of mutations in exon 3 of β-catenin gene have ranged from 0 to over 30 percent, 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 a timing of β-catenin mutation in the late stage progression in 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 using microdissection technique proved to allow sampling of pure populations of tumor cells for mutations, which may prevent false-negative results. [19] The discrepancy in frequency with previous reports could be due to 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 generation of stomach cancers in

the Mongolian gerbil model with MNU exposure and H. pylori infection but uncontrolled activation of Wnt signaling pathway could contribute stomach carcinogenesis in certain tumors. In this study, one β-catenin mutation was detected from H. pylori-infected gerbils, showing no statistical significance between MNU+H. pylori and MNU only groups (1/45 vs. 0/7, P>0.05). Thus, H. pylori infection may not enhance b-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 be happened in our model. Whether other genetic or epigenetic alteration occur in gastric cancer cells in cases lacking β-catenin mutations is an intriguing possibility warranting further research.[25, 26]

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