

Roles of cyclooxygenase-2 and microsomal prostaglandin E synthase-1 expression and β -catenin activation in gastric carcinogenesis in *N*-methyl-*N*-nitrosourea-treated K19-C2mE transgenic mice

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K19-C2mE transgenic (Tg) mice, simultaneously expressing cyclooxygenase-2 (COX-2) and microsomal prostaglandin E synthase-1 (mPGES-1) in the gastric mucosa under the cytokeratin 19 gene promoter, were here treated with *N*-methyl-*N*-nitrosourea (MNU) and inoculated with *Helicobacter pylori* (*H. pylori*) to investigate gastric carcinogenesis. Wild-type (WT) and Tg mice undergoing MNU treatment frequently developed tumors in the pyloric region (100% and 94.7%, respectively); multiplicity in Tg was higher than that in WT ($P < 0.05$) with *H. pylori* infection. Larger pyloric tumors were more frequently observed in Tg than in WT ($P < 0.05$). In addition, Tg developed fundic tumors, where WT did not. No gastric tumors were observed without MNU treatment. Transcripts of TNF- α , iNOS, IL-1 β , and CXCL14 were up-regulated with *H. pylori* infection in both genotypes and were also increased more in Tg than in WT within *H. pylori*-inoculated animals. Immunohistochemical analysis demonstrated significantly greater β -catenin accumulation in pyloric tumors, compared with those in the fundus ($P < 0.01$) with mutations of exon 3; 18.2% and 31.6% in MNU-alone and MNU + *H. pylori*-treated WT, whereas 21.4% and 62.5% was observed in the Tg, respectively; the latter significantly higher ($P < 0.05$), suggesting the role of *H. pylori* in Wnt activation. In conclusion, K19-C2mE mice promoted gastric cancer in both fundic and pyloric regions. Furthermore β -catenin activation may play the important role of pyloric carcinogenesis especially in *H. pylori*-infected Tg. Induction of various inflammatory cytokines in addition to overexpression of COX-2/mPGES-1 could be risk factors of gastric carcinogenesis and may serve as a better gastric carcinogenesis model. (*Cancer Sci* 2008; 99: 2356–2364)

There is a large body of evidence that *Helicobacter pylori* (*H. pylori*) infection is involved in development of chronic gastritis, peptic ulceration, and gastric cancer.^(1,2) Recent reports have revealed that *H. pylori* infection induces cyclooxygenase-2 (COX-2) expression and microsomal prostaglandin E synthase-1 (mPGES-1), enzymes responsible for synthesizing prostaglandin E₂ (PGE₂) in gastric mucosa.^(3–5) In the stomach, prostaglandins are major molecules for maintaining the gastric mucosa.⁽⁶⁾ PGE₂ plays distinct roles in tumor growth and metastasis in several cancers.⁽⁷⁾

Oshima *et al.* recently constructed transgenic mice (K19-C2mE) that simultaneously overexpress COX-2 and mPGES-1 in the gastric mucosa under the influence of the cytokeratin 19 gene promoter.⁽⁴⁾ The transgenic (Tg) mice develop inflammation-associated hyperplastic lesions in the proximal glandular stomach, similar to those found in the *Helicobacter*-infected stomach.⁽⁴⁾

Furthermore, K19-Wnt1/C2mE Tg mice, simultaneously expressing Wnt1 as well as COX-2/mPGES-1, develop dysplastic gastric tumors,⁽⁸⁾ indicating that COX-2/mPGES-1 and Wnt pathway activation might be involved in mouse gastric neoplasia.

The Wnt/ β -catenin signaling pathway plays important roles in cell–cell adhesion and cell cycle regulation and its alternation is implicated in genesis of many cancers. Abnormal nuclear accumulation of β -catenin due to mutation of the β -catenin gene stimulates the expression of β -catenin/Tcf target genes, such as *c-myc*, *c-jun*, and *cyclin D1*.^(9,10) Mutations of β -catenin gene exon 3, where serine and threonine residues are physiologically phosphorylated by glycogen synthase kinase (GSK)-3 β , prevent degeneration by APC/GSK-3 β /Axin complex. With human gastric cancers, nuclear accumulation of β -catenin has been estimated to occur in 12–37% of cases,^(11–15) with mutations in exon 3 reported in a few to over 20%.^(12,16,17) However, the degree of involvement of Wnt pathway alteration in the development of mouse gastric cancers remains unclear.

In the present study, Tg mice were treated with a stomach carcinogen, *N*-methyl-*N*-nitrosourea (MNU), and inoculated with *H. pylori* to investigate the influence of COX-2/mPGES-1 expression and *H. pylori* infection on mouse gastric carcinogenesis. Furthermore, we analyzed the frequency of β -catenin activation and gene mutations to assess involvement of the Wnt pathway.

Materials and Methods

Experimental design. The experimental design is shown in Fig. 1. K19-C2mE Tg mice and littermate wild-type (WT) mice were randomly divided into four groups (groups A–D). The mice of groups B and D were inoculated intragastrically with 0.8 mL of broth culture containing *H. pylori*. After 1 week, the mice of groups C and D were given MNU (Sigma Chemical Co., St Louis, MO, USA) in drinking water at the concentration of 120 p.p.m. on alternate weeks (total exposure was 5 weeks) and then normal tap water until the end of experiment. MNU was dissolved in distilled water and freshly prepared three times per week. At the end of the experiment, all surviving mice were sacrificed under deep anesthesia 60 min after an intraperitoneal

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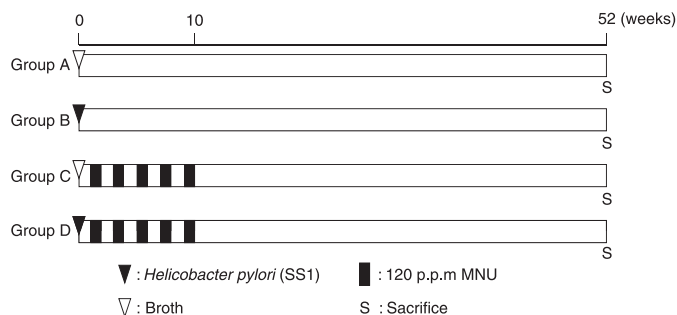


Fig. 1. Experimental design. Five- to 6-week-old K19-C2mE transgenic mice and littermate wild-type mice were inoculated with *Helicobacter pylori* SS1 (groups B and D) or broth (groups A and C). After 1 week, animals of groups C and D were administered 120 p.p.m. *N*-methyl-*N*-nitrosourea on alternate weeks (total exposure, 5 weeks).

injection of 5'-bromo-2'-deoxyuridine (BrdU) at a dose of 100 mg/kg. The excised stomachs were fixed in 10% neutral-buffered formalin or 95% ethanol plus 1% acetic acid for histology and immunohistochemistry.

Animals. K19-C2mE transgenic (Tg) mice produced by Oshima *et al.*⁽⁴⁾ were maintained by breeding male K19-C2mE Tg with female C57BL/6 N at the Animal Facility of Aichi Cancer Center Research Institute. WT mice were used as controls. All were housed in plastic cages with hardwood chips in an air-conditioned room with 12 h light–12 h dark cycle and given a basal diet (CA-1; CLEA Japan Inc., Tokyo, Japan) and water *ad libitum*. For genotyping of each mouse, DNA samples were extracted from the tails using a DNeasy tissue kit (Qiagen, Tokyo, Japan) and subjected to polymerase chain reaction (PCR) as reported elsewhere.⁽⁴⁾

Bacterial culture. *H. pylori* strain SS1 was inoculated on Brucella agar plates (Merck, Darmstadt, Germany) containing 7% v/v heat-inactivated fetal calf serum and incubated at 37°C under microaerobic conditions using an Anaero Pack Campylo (Mitsubishi Gas Chemical Co., Tokyo, Japan) at high humidity for 2 days. Then, bacteria grown on the plates were introduced into Brucella broth (Becton Dickson, Cockeysville, MD, USA) supplemented with 7% v/v fetal calf serum, and cultures of *H. pylori* were checked under a phase contrast microscope for bacterial shape and mobility.

Histopathological analysis. Tissue sections were stained with hematoxylin–eosin and Alcian blue (pH 2.5)–periodic acid-Schiff (AB-PAS) for histological analysis. Tumor location was categorized into 'fundic' and 'pyloric', the former included tumors developing in the gastric fundic mucosa and border areas of fundic and pyloric glands. The glandular mucosa was examined histologically for any inflammatory and epithelial changes. Active chronic gastritis was estimated according to criteria modified from the updated Sydney System, characterized by infiltration of neutrophils and lymphocytes. The degree of change was graded in a scale from 0 to 3, (0 [normal], 1 [mild], 2 [moderate], and 3 [marked]). Mucosa thickness was measured using AxioVision 4.6 (Carl Zeiss, Jena, Germany). Tumor size was evaluated by the largest tumor area using NIH image version 1.62 (National Institutes of Health, USA) on hematoxylin–eosin sections. Serial sections were also stained immunohistochemically with antibodies against COX-2 (Cayman Chemical, Ann Arbor, MI, USA), β -catenin (clone 14; BD Transduction Laboratories, KY, USA), and BrdU (Dako, Glostrup, Denmark). BrdU labeling index was calculated as the percentages of BrdU-positive epithelial cells within glands at five different arbitrarily selected points in gastric mucosa.

Analysis of inflammatory cytokine mRNA by quantitative real-time reverse transcription (RT)-PCR. Total RNA was extracted from the

Table 1. Primer sequences used for quantitative reverse transcription-polymerase chain reaction

Target	Primer sequence	Product size (bp)
COX-2	5'-AAGCCCTCTACAGTGACATC-3'	115
	5'-GAGAATGGTGTCCAAAGCTCTA-3'	
TNF- α	5'-GCCGATGGGTTGTACTTGTCTACT-3'	134
	5'-ACGGCAGAGAGGAGGTTGACTT-3'	
iNOS	5'-CCGGCAAACCAAGGTCTACGTT-3'	128
	5'-CACATCCCAGCCATGCGCACATCT-3'	
IL-1 β	5'-TTGACTTCACCATGGAATCCGTGTC-3'	126
	5'-GAGTCCCCTGGAGATTGAGC-3'	
IL-6	5'-CCTACCCCAATTTCCAATGCTCT-3'	143
	5'-CACTAGGTTTCCGAGTAGATCTCA-3'	
CXCL14	5'-TGTTGAGACCGTTACAGCACTAC-3'	122
	5'-GAAACTCTGACCAGTCATAAGCC-3'	
GAPDH	5'-CAACTCCCACTTCCACCTTCGAT-3'	106
	5'-CCTGTTGCTGTAGCCGTATTC-3'	

COX-2, cyclooxygenase-2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-1 β , interleukin-1 β ; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; TNF- α , tumor necrosis factor- α .

border areas of fundic and pyloric regions in the glandular stomach mucosa using an RNeasy Plus Mini kit (Qiagen, Hilden, Germany). First strand cDNAs were synthesized using a Super Script III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Relative quantitative PCR for COX-2, tumor necrosis factor- α (TNF- α), inducible nitric oxide synthase (iNOS), interleukin-1 β (IL-1 β), IL-6, and CXCL14 was performed using the mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as an internal control with the StepOne Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) using a QuantiTect SYBR Green PCR kit (Qiagen). The primer sequences are listed in Table 1. Quantification was performed as earlier established.⁽¹⁸⁾ The expression levels were expressed relative to 1.00 in WT mice in the control group A.

DNA extraction and direct sequencing. Immunoreactivity of β -catenin was classified into 'nuclear/cytoplasmic' or 'membranous' according to the intracellular localization of β -catenin protein. Tumors with 5% or more section area of nuclear/cytoplasmic β -catenin were judged as β -catenin accumulating. Tumor areas with nuclear/cytoplasmic or membranous β -catenin localization and surrounding gastric mucosa in serial paraffin sections (5- μ m thick) were microdissected using a laser microdissection system (AS LMD; Leica Microsystems, Wetzlar, Germany). Microdissection, PCR, and sequencing were performed as previously reported.^(19,20) The PCR primer sequences to amplify exon 3 of mouse β -catenin gene were 5'-AGCCACTGGCAG-CAGCAGTCTTAC-3' and 5'-ATAAAGGACTTGGGAGGTGTC-AACA-3'. Sequencing was performed using a BigDye Terminator Cycle Sequencing Kit (v 3.1; Applied Biosystems) with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Statistical analysis. The incidences of gastric tumors and frequencies of β -catenin accumulation and gene mutation were analyzed using Fisher's exact probability test. Differences of inflammation scores were assessed with the Mann-Whitney *U*-test.

Results

Incidence, multiplicity, and size of gastric tumors. The observed incidences and multiplicities of gastric tumors are summarized in Table 2. In the fundic region, Tg mice of groups C and D developed dysplastic gastric tumors (Fig. 2Aa,b), whereas WT mice did not. In contrast, WT (Fig. 2Ba,b) and Tg (Fig. 2Ca,b)

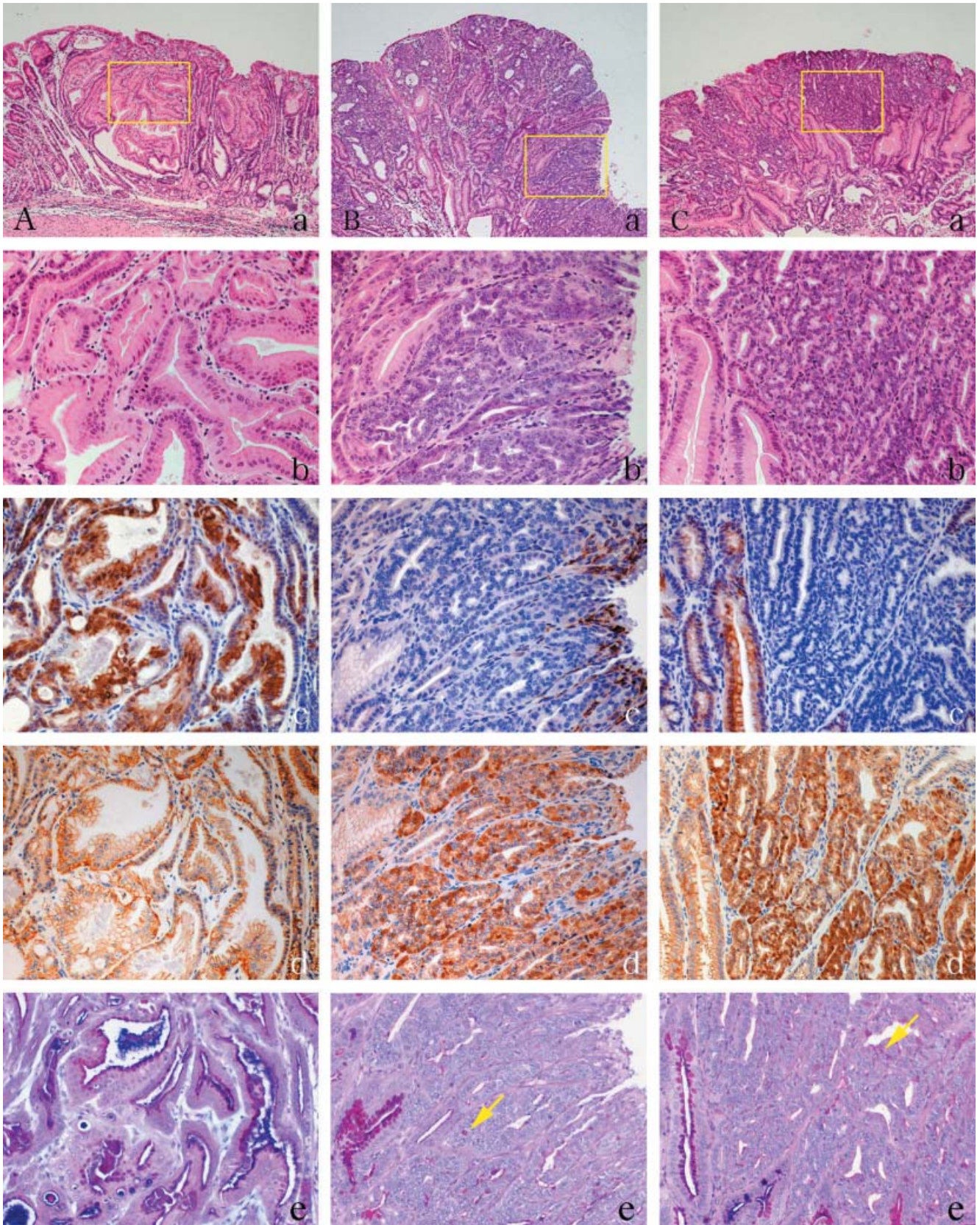


Fig. 2. Histopathological and immunohistochemical analysis of COX-2 and β -catenin in *N*-methyl-*N*-nitrosourea (MNU)-induced tumors in K19-C2mE Tg mice. (A) Gastric tumors in the fundic region of MNU-treated Tg mice. (B,C) Gastric tumors with nuclear β -catenin accumulation in the pyloric region of MNU-induced WT (B) and Tg mice (C). (a,b) Hematoxylin-eosin staining. (c,d) Immunohistochemistry for COX-2 (c) and β -catenin (d). (e) Alcian blue (pH 2.5)-periodic acid-Schiff (AB-PAS). Yellow arrow shows PAS-positive mucin. Yellow boxes in (a) are magnified in (b-e), respectively. Original magnification, 50 \times (a), 200 \times (b-e).

Table 2. Incidence and multiplicity of MNU-treated K19-C2mE mice with gastric tumors

Groups	Treatments	Genotypes	Effective nos.	Incidence (%)		Tumor multiplicity in pyloric mucosa (no. of tumors/mouse) [†]
				Fundic mucosa	Pyloric mucosa	
A	Broth	WT	10	0 (0%)	0 (0%)	0
		Tg	10	0 (0%)	0 (0%)	0
B	<i>H. pylori</i>	WT	10	0 (0%)	0 (0%)	0
		Tg	10	0 (0%)	0 (0%)	0
C	Broth + MNU	WT	12	0 (0%)	12 (100%)	1.40 ± 0.70
		Tg	15	4 (26.7%)	15 (100%)	2.00 ± 0.88
D	<i>H. pylori</i> + MNU	WT	24	0 (0%)	24 (100%)	1.75 ± 0.74
		Tg	19	4 (21.1%)*	18 (94.7%)	2.28 ± 0.96*

H. pylori, *Helicobacter pylori*; MNU, *N*-methyl-*N*-nitrosourea; Tg, Transgenic; WT, wild type.

* $P < 0.05$ versus WT within group D.

[†]Values are expressed as average ± SD.

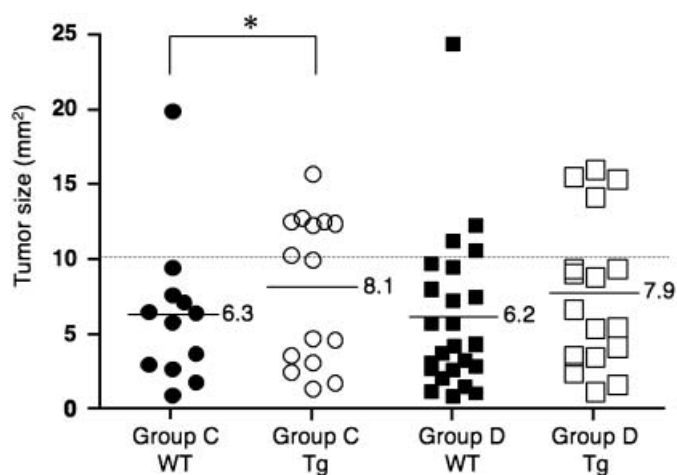


Fig. 3. Maximum tumor size in individual *N*-methyl-*N*-nitrosourea (MNU)-induced K19-C2mE mice in the pyloric region. Ratio of larger tumors (> 10.0 mm²): Tg versus WT in group C, 8/15 = 53.3% versus 1/12 = 8.3% (* $P < 0.05$); 4/24 = 16.7% versus 4/17 = 23.5% (not significant) in group D. Dotted line, cut-off value (10 mm²). Horizontal lines and values, mean.

mice of groups C and D developed dysplastic gastric tumors in the pyloric region; incidences were 100% and 94.7%, respectively, the difference not being significant among the groups and genotypes. Tumor multiplicity in Tg mice in group D (2.28 ± 0.96 tumors/mouse) was higher than those in WT mice in group C (1.40 ± 0.70) and in group D (1.75 ± 0.74) ($P < 0.01$ and $P < 0.05$, respectively). The maximum tumor size in each animal is plotted in Fig. 3. Averages ± SD were 6.3 ± 5.0 and 8.1 ± 5.0 in WT and Tg mice in group C. The corresponding figures were 6.2 ± 5.2 and 7.9 ± 5.1 in group D. The ratio of larger tumors (> 10.0 mm²) in Tg mice (8/15 = 53.3%) was significantly more frequent than that in the WT (1/12 = 8.3%) mice within group C ($P < 0.05$), but not within group D (4/24 = 16.7% and 4/17 = 23.5% in WT and Tg, respectively, $P = 0.70$). No gastric tumors were observed in groups A and B.

Status of gastritis. Data for the gastritis status in each group are summarized in Table 3. The gastric mucosa of *H. pylori* infected groups (B and D) showed significantly higher scores for infiltration of neutrophils and lymphocytes than the non-*H. pylori*-infected groups (A and C). There were no significant differences in scores for infiltration of neutrophils and lymphocytes between genotypes. Gastric mucosa of Tg mice was significantly thickened compared with that of WT.

Correspondingly, gastric mucosa of mice with *H. pylori* infection was also significantly thickened compared with mice without *H. pylori* infection. BrdU labeling indices were increased with *H. pylori* infection irrespective of genotypes (groups B and D), associated with hyperplastic change in fundic mucosa. In pyloric mucosa, the BrdU labeling index was higher in Tg mice in group D.

Alteration of expression of inflammatory cytokines. Data for the expression levels of inflammatory factors in each group are summarized in Table 4. The mRNA expressions of TNF- α , iNOS, IL-1 β , and CXCL14 in *H. pylori*-infected groups were significantly increased compared with non-*H. pylori*-infected groups in both genotypes. COX-2 was not up-regulated with *H. pylori* infection in WT animals. Furthermore, the mRNA expression levels of COX-2, TNF- α , iNOS, IL-1 β , and CXCL14 in Tg mice were significantly higher than those in WT mice in group D. In the groups A–C, there were no significant differences in expression of inflammatory factors between both genotypes except for COX-2.

Immunolocalization of COX-2 and β -catenin, and mucin staining. COX-2 expression was retained in the fundic tumor cells (Fig. 2Ac) as well as surrounding normal foveolar epithelium (Fig. 2Cc, left image). But it was attenuated in pyloric tumor cells in WT (Fig. 2Bc) and Tg (Fig. 2Cc, right image) mice. Besides the epithelial tumor cells, infiltrating and/or stromal cells also expressed COX-2 in tumor stroma (right surface area in Fig. 2Bb). β -Catenin was localized on the membrane in fundic tumors (Fig. 2Ad) as well as in normal glands. In contrast, most pyloric tumors harbored β -catenin in cytoplasm or nuclei both in WT and Tg mice (Fig. 2Bd and Cd, respectively) (Table 5). Gastric cancer cells in the pyloric region contained little PAS-positive mucin (Fig. 2Be and Ce), whereas those in fundic area retained AB- and PAS-positive mucin (Fig. 2Ae).

β -Catenin accumulation and gene mutations in gastric tumors. Data for number and frequency of fundic and pyloric tumors demonstrating β -catenin accumulation are summarized in Table 5. β -Catenin accumulation was significantly more common in tumors in pyloric mucosa compared with those in the fundic region in both WT and Tg genotypes ($P < 0.01$).

To analyze β -catenin gene mutation in gastric tumors with β -catenin accumulation, microdissection was performed for corresponding regions from serial paraffin sections. All mutations of β -catenin gene exon 3 were identified in β -catenin accumulating (nuclear/cytoplasmic) regions except for one membranous staining case in group D (Table 6). Mutations of β -catenin gene exon 3 were more frequently observed in the Tg mice of group D than in the Tg mice of group C ($P < 0.05$). Mutation frequency of β -catenin accumulating regions in WT and Tg mice in group C were 18.2% and 21.4%. The corresponding

Table 3. Histopathological response in gastric musoca of K19-C2mE Tg mice

Groups	Treatments	Genotypes	Effective nos.	Neutrophils	Lymphocytes	Fundic mucosa thickness (μm)		BrdU labeling index (%)
						Fundic mucosa	Pyloric mucosa	
A	Broth	WT	10	0.33 \pm 0.82	0.17 \pm 0.41	342.8 \pm 38.7	7.46 \pm 1.94	12.16 \pm 2.22
		Tg	10	0.18 \pm 0.40	0.27 \pm 0.47	520.8 \pm 283.0****	7.56 \pm 2.00	12.62 \pm 3.01
B	<i>H. pylori</i>	WT	10	2.70 \pm 0.48*	2.80 \pm 0.42*	593.2 \pm 111.2*	12.38 \pm 1.64*	14.97 \pm 3.18
		Tg	10	2.60 \pm 0.52*	2.70 \pm 0.48*	772.1 \pm 214.1*****	13.96 \pm 1.76*	14.33 \pm 2.69
C	Broth + MNU	WT	12	0 \pm 0	0.18 \pm 0.40	293.3 \pm 88.2	7.32 \pm 2.37	12.88 \pm 3.65
		Tg	15	0.43 \pm 0.76	0.07 \pm 0.27	438.2 \pm 162.1*****	7.70 \pm 1.45	12.86 \pm 3.11
D	<i>H. pylori</i> + MNU	WT	24	1.58 \pm 1.02**	2.00 \pm 0.83**	441.9 \pm 151.8**	13.43 \pm 7.18**	15.66 \pm 3.78
		Tg	19	2.11 \pm 0.68**	2.67 \pm 0.69***	517.5 \pm 129.2	16.95 \pm 4.23*****	16.04 \pm 5.46*****

BrdU, 5'-bromo-2'-deoxyuridine; *H. pylori*, *Helicobacter pylori*; MNU, *N*-methyl-*N*-nitrosourea; Tg, Transgenic; WT, wild type.

* $P < 0.01$ versus corresponding genotypes in group A; ** $P < 0.01$ versus corresponding genotypes in group C; *** $P < 0.01$ versus WT within group D; **** $P < 0.05$ versus WT within group A; ***** $P < 0.01$ versus WT within group B; ***** $P < 0.01$ versus WT within group C; ***** $P < 0.05$ versus Tg in group D. Values for results are expressed as averages \pm SD.

Table 4. Relative mRNA expression levels of inflammatory cytokines in gastric mucosa of K19-C2mE mice

Groups	Treatments	Genotypes	Relative mRNA expression levels (range)					
			COX-2	TNF- α	iNOS	IL-1 β	IL-6	CXCL14
A	Broth	WT	1.00 (0.55–1.81)	1.00 (0.49–2.02)	1.00 (0.58–1.73)	1.00 (0.31–3.20)	1.00 (0.38–2.63)	1.00 (0.52–1.93)
		Tg	1824.28 (1448.04–2298.28)*****	0.68 (0.37–1.24)	0.68 (0.18–2.52)	0.97 (0.37–2.49)	1.22 (0.64–2.29)	0.76 (0.41–1.43)
B	<i>H. pylori</i>	WT	2.07 (0.76–5.64)	27.74 (16.21–47.46)*	18.85 (8.56–41.49)*	31.45 (20.52–48.20)*	2.74 (1.83–4.10)**	3.96 (2.94–5.34)*
		Tg	1272.96 (694.86–2332.02)*****	42.98 (15.66–117.96)*	38.59 (9.57–155.70)*	32.41 (14.04–74.81)*	2.55 (2.08–3.14)*	5.12 (3.25–8.06)*
C	Broth + MNU	WT	2.00 (1.18–3.39)	5.61 (2.55–12.32)*	4.23 (2.92–6.12)*	8.42 (4.35–16.30)*	1.23 (0.35–4.27)	4.94 (2.21–11.07)*
		Tg	2333.23 (1588.37–3427.38)*****	4.81 (2.75–8.43)*	5.98 (3.11–11.51)*	9.61 (4.73–19.55)*	1.59 (0.13–19.12)	6.35 (3.35–12.03)*
D	<i>H. pylori</i> + MNU	WT	2.18 (1.21–3.91)	26.63 (8.78–80.76)****	19.08 (5.13–71.02)****	25.18 (10.67–59.45)****	3.22 (1.61–6.44)	4.53 (2.42–8.47)***
		Tg	901.64 (492.31–1651.30)*****	61.19 (20.55–182.24)*****	42.31 (12.94–138.40)*****	54.03 (19.62–148.80)*****	3.74 (1.29–10.85)	8.04 (5.09–12.71)*****

COX-2, cyclooxygenase-2; *H. pylori*, *Helicobacter pylori*; IL-1 β , interleukin-1 β ; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; MNU, *N*-methyl-*N*-nitrosourea; Tg, Transgenic; TNF- α , tumor necrosis factor- α ; WT, wild type.

* $P < 0.01$ or ** $P < 0.05$ versus corresponding genotypes in group A; *** $P < 0.01$ versus corresponding genotypes in group B; **** $P < 0.01$ or ***** $P < 0.05$ versus corresponding genotypes in group C; ***** $P < 0.01$ versus WT in same groups. Values are expressed as mean. Ranges in parenthesis are expressed as mean – SD to mean + SD.

Table 5. Number and frequency of β -catenin accumulation in fundic and pyloric tumors

Groups	Treatments	Genotypes	Number (frequency) of β -catenin accumulation	
			Fundic mucosa	Pyloric mucosa
C	Broth + MNU	WT	NA	12/12 (100%)
		Tg	0/4 (0%)	15/15 (100%)*
D	<i>H. pylori</i> + MNU	WT	NA	22/24 (91.6%)
		Tg	1/4 (25%)	18/18 (100%)**

H. pylori, *Helicobacter pylori*; MNU, *N*-methyl-*N*-nitrosourea; NA, not applicable due to no tumors; Tg, Transgenic; WT, wild type.
P* < 0.01 versus that of fundic mucosa in Tg within group C; *P* < 0.01 versus that of fundic mucosa in Tg within group D.

figures in group D were 31.6% and 62.5%, respectively. All mutations observed in pyloric tumors were transitions: C→T (9/22 = 41%), G→A (9/22 = 41%), T→C (4/22 = 18%) (Table 7). No mutations were detected in surrounding normal mucosa.

Discussion

In the present study, K19-C2mE Tg mice developed gastric tumors not only in the pyloric mucosa but also the fundic region, whereas WT mice developed tumors only in pyloric areas with the carcinogen treatment but irrelevant to *H. pylori* infection. Furthermore, Tg mice possessed larger tumors even without *H. pylori* infection. On the other hand, Tg mice showed increased tumor multiplicity compared with the corresponding WT mice only with *H. pylori* infection. These findings indicated that transgenes in combination with *H. pylori* infection and subsequent inflammatory response should play important roles in promotion of gastric carcinogenesis in various ways in this mouse model.

Immunohistochemical analysis here demonstrated that tumor cells in the fundic region more markedly express COX-2 than those in the pyloric region. COX-2 expression was predominantly observed in foveolar epithelial cells in K19-C2mE Tg mice. Thus fundic tumors might be derived from foveolar epithelial cells and be more significantly affected by COX-2 expression compared with pyloric tumors. In human gastric neoplasia, proximal gastric tumors are suggested to be specific subtypes of gastric carcinoma based on histological and genetic research.^(21,22) K19-C2mE Tg mice may serve as a new animal model for proximal gastric carcinogenesis.

In the present study, *H. pylori* infection did not promote gastric carcinogenesis in the fundic region, and influence could not be evaluated in the pyloric region in terms of cancer incidence. However, there is abundant evidence from rodent gastric cancer models that *H. pylori* infection promotes gastric cancers induced by stomach carcinogens, MNU and *N*-methyl-*N'*-nitroso-*N*-nitrosoguanidine (MNNG),⁽²³⁻²⁷⁾ although not without exceptions.⁽²⁸⁾ *H. pylori* infection induced gastritis and caused hyperplasia of the gastric mucosa in the current mouse system, but heterotopic proliferating glands arising with long-term infection of *H. pylori* and considered as high-grade inflammation in the Mongolian gerbil,^(29,30) were not observed here. Such lesions are reversible and are considered as regenerative lesions due to excessive cell proliferation. The observations indicate that the influence of *H. pylori* infection may depend on the animal species with clear differences between the mouse and Mongolian gerbil. In addition, host immune responses or *H. pylori* virulence factors may affect gastritis and gastric carcinogenesis.^(31,32) By the fact of increased multiplicity of pyloric tumors in *H. pylori*-infected Tg mice, overexpression of COX-2 and mPGES-1 may serve as a better mouse model for mimicking human cases.

Table 6. Localization of β -catenin in gastric tumors and mutation frequency

Groups	Treatments	Genotypes	Animal nos.	Frequency of mice with mutations	β -Catenin mutation frequency				Surrounding normal mucosa
					in fundic tumors		in pyloric tumors		
					Nuclear + Cytoplasmic	Membranous	Nuclear + Cytoplasmic	Membranous	
C	Broth + MNU	WT	10	2/10 (20%)	NA	NA	2/11 (18.2%)	0/7 (0%)	0/7 (0%)
		Tg	10	2/10 (20%)	NA	0/4 (0%)	3/14 (21.4%)	0/10 (0%)	0/6 (0%)
D	<i>H. pylori</i> + MNU	WT	10	4/10 (40%)	NA	NA	6/19 (31.6%)	0/10 (0%)	0/7 (0%)
		Tg	10	8/10 (80%)*	0/1 (0%)	0/4 (0%)	10/16 (62.5%)***	1/14 (7.1%)	0/7 (0%)

H. pylori, *Helicobacter pylori*; MNU, *N*-methyl-*N*-nitrosourea; NA, not applicable due to no tumors; Tg, Transgenic; WT, wild type.
P* < 0.05 versus Tg in group C; *P* < 0.05 versus Tg in 'membranous' in within group D.

Table 7. Mutation of β -catenin exon 3 in gastric tumors

Groups	Treatments	Genotypes	Mice nos.	Sample nos.	Tumor location	β -Catenin localization	Mutations	Amino acid changes	Events
C	Broth + MNU	WT	W-6	T4	Pylorus	Nuclear/Cytoplasmic	codon 41: ACC→ATC	Thr→Ile	Transition
			W-11	T9	Pylorus	Nuclear/Cytoplasmic	codon 34: GGA→GAA	Gly→Glu	Transition
		Tg	T-1	T12	Pylorus	Nuclear/Cytoplasmic	codon 32: GAT→AAT	Asp→Asn	Transition
				T13	Pylorus	Nuclear/Cytoplasmic	codon 32: GAT→AAT	Asp→Asn	Transition
				T-12	T22	Pylorus	Nuclear/Cytoplasmic	codon 34: GGA→GAA	Gly→Glu
D	<i>H. pylori</i> + MNU	WT	W-8	T27	Pylorus	Nuclear/Cytoplasmic	codon 32: GAT→AAT	Asp→Asn	Transition
			W-11	T75	Pylorus	Nuclear/Cytoplasmic	codon 35: ATC→ATT	Silent	Transition
				T75	Pylorus	Nuclear/Cytoplasmic	codon 45: TCC→TTC	Ser→Phe	Transition
				T85	Pylorus	Nuclear/Cytoplasmic	codon 41: ACC→ATC	Thr→Ile	Transition
			W-24	T34	Pylorus	Nuclear/Cytoplasmic	codon 33: TCT→CCT	Ser→Pro	Transition
				T35	Pylorus	Nuclear/Cytoplasmic	codon 33: TCT→CCT	Ser→Pro	Transition
				T-2	T37	Pylorus	Nuclear/Cytoplasmic	codon 41: ACC→ATC	Thr→Ile
			Tg	T38	Pylorus	Nuclear/Cytoplasmic	codon 41: ACC→ATC	Thr→Ile	Transition
				T39	Pylorus	Membranous	codon 41: ACC→ATC	Thr→Ile	Transition
		T-4		T40	Pylorus	Nuclear/Cytoplasmic	codon 32: GAT→AAT	Asp→Asn	Transition
		T41		Pylorus	Nuclear/Cytoplasmic	codon 41: ACC→ATC	Thr→Ile	Transition	
		T-5		T88	Pylorus	Nuclear/Cytoplasmic	codon 32: GAT→AAT	Asp→Asn	Transition
		T-6		T90	Pylorus	Nuclear/Cytoplasmic	codon 33: TCT→CCT	Ser→Pro	Transition
		T-16		T158	Pylorus	Nuclear/Cytoplasmic	codon 32: GAT→AAT	Asp→Asn	Transition
		T-17	T147	Pylorus	Nuclear/Cytoplasmic	codon 32: GAT→AAT	Asp→Asn	Transition	
		T-18	T92	Pylorus	Nuclear/Cytoplasmic	codon 41: ACC→ATC	Thr→Ile	Transition	
		T-19	T149	Pylorus	Nuclear/Cytoplasmic	codon 41: ACC→ATC	Thr→Ile	Transition	

H. pylori, *Helicobacter pylori*; MNU, *N*-methyl-*N*-nitrosourea; Tg, Transgenic; WT, wild type.

For further analysis of factors promoting gastric carcinogenesis, we investigated the expression of inflammatory cytokines in gastric mucosa. In the present study, expression of those such as TNF- α , iNOS, IL-1 β , and CXCL14 were significantly increased in Tg mice with *H. pylori* infection and MNU treatment. Among them, CXCL14 has been known to selectively attract monocytes, where PGE₂ up-regulates their responsiveness.⁽³³⁾ The combination of these factors may contribute to the participation of macrophages in increased tumorigenesis in Tg mice with *H. pylori* infection plus MNU treatment. We previously demonstrated that the severity of chronic gastritis, characterized by high-level expression of IL-1 β , TNF- α , COX-2, and iNOS was concerned with glandular gastric carcinogenesis in *H. pylori*-infected Mongolian gerbils.⁽³⁴⁾ Thus, in Tg mice with *H. pylori* infection a higher level of inflammatory cytokines may be induced that eventually promotes gastric carcinogenesis.

The Tg mice feature increased PGE₂ synthesis due to over-expressed COX-2/mPGES-1 in gastric mucosa.⁽⁴⁾ PGE₂ exerts its biological effects by binding to four isoform receptors, EP₁, EP₂, EP₃, and EP₄,^(35,36) categorized in the family of seven transmembrane G protein coupled rhodopsin-type receptors. Accumulating evidence indicates that PGE₂ promotes tumor growth by stimulating EP receptor signaling with subsequent enhancement of cell proliferation, promotion of angiogenesis, and inhibition of apoptosis.⁽³⁷⁾ Previous reports based on mouse studies demonstrated that EP₁, EP₂, and EP₄ receptors play important roles in colon carcinogenesis.⁽³⁸⁻⁴¹⁾ Furthermore, expression of EP₁, EP₂, and EP₄ receptors has been found to be elevated in mouse mammary tumors as well as colon cancers.⁽⁴²⁾ EP₃ receptor activation was furthermore suggested to contribute to breast cancer progression.⁽⁴³⁾ These observations indicate that the expression pattern of EP receptors in cancer cells might determine the potential of PGE₂ to drive tumor progression. Although the expression level of EP receptors in gastric cancers was unclear in the present study, EP receptor signaling stimulated by PGE₂ might have influenced gastric carcinogenesis. It should be stressed that PGE₂ transactivates epidermal growth factor receptor

(EGFR) and triggers PI3K/Akt signaling,⁽⁴⁴⁻⁴⁶⁾ and Ras/MEK/ERKs,⁽⁴⁷⁾ pathways in gastric epithelial and colon cancer cells *in vitro* as well as rat gastric mucosa *in vivo*.⁽⁴⁶⁾

To analyze the differences between present mouse gastric tumors developed in fundic and pyloric mucosas, we investigated β -catenin activation, which was suggested to play an important role in gastric carcinogenesis. Immunohistochemical analysis here showed that β -catenin activation characterized by its intracellular accumulation was frequently observed in tumors in the pyloric region, in contrast to those in the fundic region, indicating the unnecessary of the Wnt pathway. Conversely, pyloric tumorigenesis might be promoted by Wnt activation. In a rat model, a type of adenocarcinoma resembling foveolar epithelium showed nuclear accumulation of cyclin D1 without β -catenin activation,⁽⁴⁸⁾ whereas β -catenin was accumulated in cytoplasm or nuclei in the majority of less-differentiated adenocarcinomas.⁽²⁰⁾ Since fundic tumors were histologically classified as being of the foveolar type and pyloric ones were classified as the less differentiated in this experiment, oncogene activation could depend on cell/tissue differentiation or vice versa.⁽⁴⁹⁾ Similarly, mucin expression characterized by AB-PAS staining was observed in fundic tumors as in the hyperplastic fundic tumors in the previous report;⁽⁴⁾ pyloric tumors, however, lost most of those mucin production.

Several previous studies of gastric carcinogenesis models in rodents such as the mouse, rat, and Mongolian gerbil have indicated that β -catenin activation plays an important role in gastric carcinogenesis.^(20,50-52) In the present study, this was more frequently observed than in previous reports (rat, 18.2%; mouse, 12.5%; and Mongolian gerbil, 2.2%).^(20,50,51) Such variation in the frequency of β -catenin activation might be caused by differences in experimental design such as the experimental period, chemical carcinogen applied, or type of experimental animal.

In rat and Mongolian gerbil models, mutations of β -catenin gene in exon 3 have been identified in codons 34, 41, and 45 at GSK-3 β phosphorylation sites, and are significantly associated with nuclear β -catenin accumulation.^(20,51) In human gastric cancers,

gene alternations have been found in the same sites including codons 29, 37, 41, and 47 as well as in adjacent sites at codons 28, 32, 34, 36, 38, 39, and 48.^(12,13,19,53) In the present experiment, the mutation spectrum was codons 32, 33, 34, 35, 41, and 45, consistent with the previous reports. Furthermore, β -catenin mutations were particularly frequent in Tg mice with *H. pylori* infection. Tumor multiplicity was also increased in Tg mice with *H. pylori* infection, indicating the possibility that *H. pylori* infection in addition to COX-2/mPGES-1 expression might contribute to progression of gastric adenocarcinomas through β -catenin gene alternations at least in part. In humans, stomach cancers with intestinal differentiation markers feature more β -catenin mutations compared to those with gastric markers.⁽¹⁹⁾ Furthermore, intestinal markers may be induced only in stomach tumors in *H. pylori*-infected gerbils.⁽⁵⁴⁾ Thus, alteration of β -catenin could be related to *H. pylori* infection, although further work is needed to reveal interactions between these two factors.

β -Catenin accumulation without β -catenin gene mutations was detected in some tumors in the present study, indicating involvement of other alterations of Wnt pathway regulatory genes. Indeed, there have been a large number of previous reports suggesting that *APC* gene mutation,^(17,55-61) *APC* loss of heterozygosity,⁽⁵⁹⁾ over-expression of various Wnt ligands, and altered frizzled receptors⁽⁶²⁻⁶⁴⁾ may be involved in β -catenin

activation. Furthermore, degradation of E-cadherin and microsatellite instability might also be responsible for β -catenin accumulation. In one rodent model, the *APC*^{Min/+} mouse which harbors a nonsense mutation at codon 850 of the *APC* gene, gastric tumors frequently develop with *APC* loss of heterozygosity.⁽⁵⁰⁾

In conclusion, the present study indicated that over-expression of COX-2/mPGES-1 promotes gastric carcinogenesis, especially in the fundic region, further showing the K19-C2mE Tg mouse to be a new animal model for proximal gastric carcinogenesis. Furthermore Tg mice developed multiple tumors in the pyloric region with *H. pylori* infection partly with β -catenin gene mutation and activation. This indicates the risk of multiple or meta-chronous gastric cancers also in human cases with *H. pylori* infection and supports the idea to eradicate the bacterium or to suppress inflammatory response for the prevention of secondary malignancies.⁽⁶⁵⁾

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